

## **METHODS AND COMPOSITIONS FOR ELUCIDATING PROTEIN EXPRESSION PROFILES IN CELLS**

**[0001]** This application is a continuation-in-part of U.S. patent application No. 09/811,842 which claims benefit under 35 U.S.C. § 119(e) of provisional application No. 60/190,678 filed Mar. 20, 2000. This application also claims priority under 35 U.S.C. § 119(e) from provisional application 60/458,152 filed March 27, 2003. All of the foregoing applications are incorporated herein by reference in their entirety.

### **FIELD OF THE INVENTION**

**[0002]** This invention relates generally to the field of functional genomics. The invention enables the direct correlation of genomic DNA to rapidly quantifiable protein expression levels enabling the detection of a protein expression profile for a particular cell or cell type. This information can then be used to correlate with reference cells to identify differences in protein expression patterns that are responsible for differentiation, disease states, age, or any other temporal or spatial protein expression difference in particular cells for diagnosis, pathway regulation or drug target candidates.

### **BACKGROUND OF THE INVENTION**

**[0003]** The last quarter of a century has been marked by a relentless drive by molecular biologists to decipher first genes and then entire genomes. Genomics, the use of genetic and molecular biology techniques to develop complete genome maps, as well as underlying genomic sequences for different organisms, has provided an explosion of information about the underlying genes which make up all living things. The fruits of this work already include the genome sequences of 599 viruses and viroids, 205 naturally occurring plasmids, 185 organaros, 31 new bacteria, 7 archea, 1 fungus, 2 animals and 1 plant (Nature, 409:860-921 (2001) The Human Gene Consortium). A significant milestone in the field of genomics culminated recently with the announcement that the entire human genome had been sequenced.

**[0004]** The most important application of this sequence data, however will be the ultimate identification of protein coding genes. Proteins are not produced directly from DNA, instead information in the form of DNA is transcribed to form messenger RNA (mRNA) molecules. These mRNA molecules function as templates for protein synthesis (translation). Each cell in the body contains the entire genome of the organism, however, only a portion of any cell's genome is expressed at any given time. Differences in expression profiles account for the different types of cells and tissues within an organism and for a cell's varying response to stress or disease.

**[0005]** Thus, cells in different tissue in the human body are unique because they have different native genes. For example, blood cells and muscle cells not only look different, they also perform different functions. Blood cells supply oxygen to organs and protect us from disease, while muscle cells enable us to move and digest food. These differences are due to specific gene products that are unique to blood or muscle cell proteins. The presence of different proteins within the same cell is the result of the function of different genes. An example could be the generation of Ab diversity or viruses.

**[0006]** Functional genomics is aimed at discovering the biological function of particular genes and uncovering the means by which sets of genes and their products work together in health and disease. According to the Human Gene Consortium group, there appear to be about 30,000 to 40,000 protein coding genes in the human genome. Amazingly this is only about twice as many as in *C. elegans* or *D. melanogaster* the fruit fly. Thus the vast complexity of the human must be due to more complicated use of the existing genes with alternative splicing rather than simply increased number of genes.

**[0007]** If genes encode multiple proteins, then the architect of biological complexity distinguishing our genetic material from that of a worm is RNA, the molecule that directs the production of proteins from DNA. Unlike genes in bacteria, genes in plant and animal cells are not arranged as continuous DNA but as coding exons interspersed with noncoding introns making it possible to transcribe one gene into several different products as each mRNA is spliced together to form combinations of exons and bits and pieces of introns.

[0008] Previous estimates were that around 20% of human genes are transcribed in more than one alternative variant, but recent research puts the number closer to 50% and even this estimate has been criticized as conservative. For example, a team of American researchers studying genes that control brain development in *Drosophila melanogaster* reviewed calculations indicating that the Neurexin genes can give rise to 35,000 different possible protein products just from alternative splicing. If you add the possibilities for RNA editing as well as post translational modifications, one could potentially end up with millions of different gene products. In fact, studies of fly species that have evolved separately for millions of years show that sequence of many alternative splice sites are strictly conserved indicating that they are in fact used.

[0009] Thus the desired endpoint for the description of a biological system is not the analysis of mRNA transcript levels alone but also the accurate measurement of protein expression levels and their respective activities. Quantitative analysis of global mRNA levels is the current method for the analysis of the state of cells and tissues, (Fraser, et al, 1997 "Strategies for whole microbial genome sequencing and analysis" Electrophoresis 18:1207-1216). Several methods have been refined to provide absolute mRNA or relative mRNA levels in comparative analysis. mRNA based genomics, however provide several inherent limitations. For example, gene (mRNA) expression levels may not always accurately predict the protein expression levels. Therefore gene expression analysis such as with micro arrays may not provide definitive information on certain targets. In fact Gygi et al., recently concluded that the correlation for all yeast proteins between mRNA and protein expression levels was less than 0.4. Indeed, for some genes, while the mRNA levels were of the same value the protein levels varied by more than 20-fold. Conversely, invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that varied by as much as 30-fold. Gygi et al. "Correlation between Protein and mRNA Abundance in Yeast" Molecular and Cellular Biology March 1999 pp 1720-1720.

[0010] Further, post translational modification of proteins such as proteolytic cleavage, glycosylation, phosphorylation, prenylation, myristoylation, ubiquitination and N- and C-terminal processing can affect protein activity and half life. These modifications cannot be determined solely from gene sequence or expression data. Some

proteins are active only when they are complexed to other molecules or proteins, or at a particular sub-cellular location within a cell. Again these factors cannot be determined from gene sequence expression data. FIG. 20 is a diagram demonstrating the layers of information which may be assayed to identify the real state of cell (furthest outward circle). Those who assay DNA and raw sequence data determine gene function based on sequence similarity, gene structure, and evolutionary relationships. Missing from this data is any mRNA or translational modification data. Those who assay mRNA gain a prediction of a protein profile based on the assumption that protein levels are directly proportional to mRNA. An assumption which is proving to be erroneous. Closest of all these methods to the real cell state is the method of the invention which detects actual cellular protein levels by direct measurement.

**[0011]** The field of study of proteomics has gained increasing importance as functional genomics attempts to assign functions to the mass of information from the human genome. Proteomics includes the science and processes of analyzing and cataloging all the proteins encoded by a genome (a proteome).

**[0012]** Complete descriptions of proteins including sequence structure and function will substantially aid the current pharmaceutical approach to therapeutics development. Thus the specific structural and functional aspects of a particular protein can be used to design better proteins or small molecule ligands that can serve as activators or inhibitors of protein function to develop drugs. Genome sequence information, due to the multitude of steps between gene transcription and corresponding protein function, is often insufficient to explain disease mechanisms.

**[0013]** Multiple genes may be involved in a single disease process. Identifying all the genes involved in a particular disease based on DNA sequence data may be possible but learning how these genes function in health and disease (and health therapeutic interventions can be designed for them) requires proteomics.

**[0014]** Disease may be caused by changes in gene expression, protein expression, or post translational modification of proteins. Many proteins are the intermediate targets for drugs, drug related changes in gene expression levels, or an indirect result of the drugs interaction with the protein. Cells and their proteomes are dynamic. One genome

may yield multiple proteomes as a result of changes in differentiation, stress, or disease condition. Proteomics can be used to determine serum based biomarkers which can be valuable as clinical markers or used as a basis of a diagnostic.

**[0015]** As can be seen, a need exists in the art for identifying proteins and their concomitant coding sequences that are directly or indirectly regulated and involved in differential expression patterns associated with disease states, different tissue types, or other alternative cell states.

### **BRIEF SUMMARY OF THE INVENTION**

**[0016]** The present invention relates generally to methods and compositions for the identification of differential protein expression patterns and concomitantly the active genetic regions that are directly or indirectly involved in different cell types, tissue types, disease states, or other cellular differences desirable for diagnosis or for drug therapy targets.

**[0017]** According to the invention a method for obtaining a protein profile in a cell is disclosed by use of a genetic integration polynucleotide encoding a tag protein which may be actively detected. The polynucleotide construct comprises a marker gene or tag which is introduced into the genome of an organism using any vector insertion method known in the art, developed in the future, or described herein. The marker gene is not operably linked to any promoter sequence in the construct (promoterless) and the construct thus relies upon integration within an active transcription unit within the cell for expression. The activity of the tag is then measured to sort and preferably quantify protein expression patterns for the cell. Once a profile expression pattern is obtained, molecular biology techniques are employed to ascertain the particular genetic loci which is expressed. This information elucidates diagnostic profiles for disease or other cellular states or types as well as elucidating potential target sites for drug intervention and alternative gene forms (SNPs). FIG. 3 depicts a general overview of the process of the invention as applied to a cancer versus a normal cell.

**[0018]** Polynucleotides for achieving the methods of the invention are disclosed including expression constructs, molecular biology techniques, transformed cells, vectors,

and methods of design of the same which are intended to be within the scope of the invention.

**[0019]** It is an object of the present invention to provide an immediate linking of protein information to its corresponding genome sequence to provide information for diagnostic protocols, pathway elucidation, or targets for drug design.

**[0020]** It is another object of the present invention to identify a protein expression profile of any particular cell, whether plant, bacterial, animal, etc. in origin, and to quantify relative levels of expression of those proteins associated with a particular population.

**[0021]** It is yet another object of the present invention to provide a library of functional genomic data that may be used to develop human therapeutics. Most researchers involved in the field of functional genomics rely on machine-based analysis for protein structure or function to assign functions to proteins. Those approaching the task from a sequencing objective assign function by analyzing and comparing genomic data developed by comparisons between disease and normal tissues. Typically this is accomplished through the use of gene chips or direct sequencing.

**[0022]** It is yet another object of the invention to provide an immediate link between genomic sequences to proteomic information using molecular biology techniques. Results of the information according to the invention can provide new therapeutic target development. Individual variations in protein expression levels between normal and aberrant tissues will lead to the direct identification of new therapeutic targets. If an unidentified protein is either higher or lower in expression levels within the malignant cells compared to the normal cells, it provides a probable target for further study and identifies a potential drug intervention site. Unique protein targets will be identified according to the invention.

**[0023]** It is yet another object of the invention to provide information about entire pathways of protein regulation, proving new target development when multiple proteins are involved in a particular state. Most cancer and other therapeutic drug development focuses on a single protein target at one time. However complex interactions between proteins result in the malignant or disease state in almost all cells. According to the

invention, applicants method identifies protein expression levels for an entire pathway of active protein targets and can evaluate expression of multiple proteins simultaneously providing for analysis of pattern of protein co-expression with malignant or disease state cells as compared to normal cells.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0024]** FIG. 1 is a schematic of a vector useful for the invention. In this example, integration of a marker peptide coding sequence can occur either in an intron or exon in split genes encoding protein products (inclusive but not limited, e.g. genes without introns that encode proteins such as histones etc., or genes encoding physiologically active RNAs, *e.g.*, snRNA, scRNA, spliceosome components etc.). For the sake of clarity, integration into an intron sequence of a cellular gene encoding a protein is shown. Placement of a splicing acceptor (SA) upstream of a marker peptide-encoding sequence results in the synthesis of a mRNA encoding a fusion protein that includes the marker peptide fused to peptide sequences encoded by upstream exons (occurs when the splice donor of the nearest upstream exon (closer to the start of transcription) is reacted to the splice donor present in the integrated marker DNA sequence).

**[0025]** FIGS. 2A-2K depict diagrams of several variant constructions of retroviral vectors which perform certain distinct functions for acquiring different types of information in cells. The critical portion is the area located between the 5' and 3' LTR. These expression cassettes would be moved essentially intact between any of the various viruses and/or plasmids that we have mentioned. FIG. 2A is a vector for exon acquisition. FIG. 2B depicts a vector designed for integration site acquisition. FIG. 2C depicts a vector for incorporation of multiple marker genes. FIG. 2D depicts a transfection cassette. FIG. 2E depicts a vector for replication compliant virus. FIG. 2F depicts a vector for a fusion protein marker for cell pre-separation and FACS analysis. RE (Type IIS restriction enzyme site); LTR, (long terminal repeat); CMV IE, (CMV intermediate early promoter); NeoR; (neomycin resistant gene); pA, (bovine growth hormone poly-A signal); SA, (human  $\gamma$ -globin intron #2 splicing acceptor); pA, NeoR, CMV, hrGFP, SA are in anti-sense orientation against LTRs. Gag, pol, env, retroviral helper virus. FIG. 2H depicts examples of HIV-1 based vectors that do not have selection markers and have the

exon trapping marker hrGFP or AcGFP flanked by either a splice acceptor site only (pHSG) or by both splice acceptor and donor signals (pHSGEX, pHS2GEX3, pHS2AcGEX3 and pHS3GEX4) which are respectively flanked by flexible linker/s of glycine and serines (GC linkers) on the amino terminus only (pHSGEX) or on both amino and carboxyl terminus of the markers hrGFP (pHS2GEX3) or AcGFP (pHS2AcGEX3, pHS3AcGEX4). Vector pHS3AcGEX4 contains an ampicillin resistance gene and a pUC bacterial origin of replication, that allows to perform rescue of the genomic sequence tags flanking the 3'LTR chromosomal insertion site by digestion with RsaI and self-ligation of chromosomal DNA followed by transformation into bacteria and selection for Amp resistance. FIG. 2I shows HIV-1 based vectors that in addition to the gene trapping exon markers hrGFP or AcGFP flanked by GC linkers, contains an expression cassette for selection of transduced cells. The expression cassettes consist of either a PGK promoter or an adenovirus Elb promoter driving the expression of either murine  $\alpha(1,3)$  galactosyl transferase ( $\alpha$ Gal) or neomycin phosphotransferase (Neo) genes. Expression of  $\alpha$ Gal results in the expression of the  $\alpha$ -galactosyl epitopes on glycoproteins and glycolipids present on the external cell surface, which allows to magnetically select for cells showing expression of this gene by using antibodies against  $\alpha$ -galactosyl residue, which is normally absent in untransduced human cells. The  $\alpha$ Gal and Neo genes are cloned in a poly-A trapping configuration to select for those insertion events that occur within transcriptional units. Vectors pHS3AcGEX4PA, pHS3AcGEX4E1bA and pHS3AcGEX4E1bNeo contain an ampicillin resistance gene and a pUC bacterial origin of replication that allows rescuing the genomic insertion sites flanking the 3'SIN LTR as described above. FIG. 2J shows four vectors that contain a gene trapping exon encoding three copies of the influenza haemagglutinin HA epitopes. Vectors pHS2HA3Xf0, pHS2HA3Xf1 and pHS2HA3Xf2 encode three copies of the HA epitope in translational frames 0, 1 and 2, respectively. Vector pHS2HA3F encodes three copies of the HA epitope, each one in a different translational frame. This allows for magnetic selection of cells showing gene trapping events that result in membrane proteins displaying the HA epitope in the external surface of the cell membrane. FIG. 2K depicts examples of vectors based on MoMLV backbones where the translation frame of the fluorescent marker protein is shifted by zero, one or two nucleotides inserted after the



splice acceptor signal. More structural features of these and other vectors are described in Table 1.

**[0026]** FIG. 3 delivers a rudimentary overview of the process of the invention. The process begins with at least two different populations of cells to be compared. Each population of cells to be compared will have been marked genetically by a vector containing marker/s-peptides to facilitate detection and determination of relative concentration of marker/s. Left portion of middle panel demonstrates separation of populations of cells based on relative amount of marker present in the tagged cells. Sequences flanking the vector will be determined by but not limited to 5' Serial Analysis of Viral Integration (5'SAVI), Serial Analysis of Viral Integration (SAVI) or inverse PCR procedure for recovering genomic tags associated to vector or viral integration events. Valid tags will then be compared to public and commercial databases and annotated into our own data bases.

**[0027]** FIG. 4 is a depiction of a gene trap vector, pGT5A with a humanized renilla fluorescence protein (hrGFP) as an assay marker, or reporter gene. (A) Schematic diagram of pGT5A plasmid. LTR, long terminal repeat; PBS, retroviral primer binding site; CMV IE, CMV intermediate early promoter; NeoR; neomycin resistant gene; pA, bovine growth hormone poly-A signal; SA, human  $\gamma$ -globin intron #2 splicing acceptor; AmpR, ampicillin-resistant gene for bacterial cloning. pA, NeoR, CMV, hrGFP, SA are in anti-sense orientation against LTRs. (B) Schematic order of genes in pGT5A vector.

**[0028]** FIG. 5 is a depiction of a vector, pGT5AH with a humanized renilla fluorescence protein (hrGFP) as an assay marker, or reporter gene. (A) Schematic diagram of pGT5AH plasmid. LTR, long terminal repeat; PBS, retroviral primer binding site; CMV IE, CMV intermediate early promoter; NeoR; neomycin resistant gene; pA, bovine growth hormone poly-A signal; SA, human  $\gamma$ -globin intron#2 splicing acceptor; AmpR, ampicillin-resistant gene for bacterial cloning. pA, NeoR, CMV, hrGFP, SA are in anti-sense orientation against LTRs. His6 tag contains 6 continuous histidine residue at c-terminal of hrGFP for detection by anti-His6 antibody. (B) Schematic order of genes in pGT5AH vector.

[0029] FIG. 6 is a depiction of pGT5Z with a humanized renilla fluorescence protein (hrGFP)) as an assay marker, or reporter gene and Zeocin-resistance gene (ZeoR). (A) Schematic diagram of pGT5Z plasmid. LTR, long terminal repeat; PBS, retroviral primer binding site; CMV IE, CMV intermediate early promoter; NeoR; neomycin resistant gene; pA, bovine growth hormone poly-A signal; SA, human  $\gamma$ -globin intron#2 splicing acceptor; SD, synthetic splicing donor. SV40, simian virus type 40 early promoter. AmpR, ampicillin-resistant gene for bacterial cloning. pA, NeoR, CMV, hrGFP, SA are in anti-sense orientation against LTRs. (B) Schematic order of genes in pGT5Z vector.

[0030] FIG. 7 is a depiction of a demonstration of the splicing function and fusion hrGFP protein expressed by pGT5A vector. (A) A construct of pGT5Z, which derived from pGT5A with an insertion of a SV40 early promoter (SV40), Zeocin-resistant gene (ZeoR), and a synthetic splicing donor and partial intron to demonstrate the expected biological functions of pGT5A after gene trapping. (B) pGT5Z-transfected cells after Zeocin selection showed significant Zeocin-hrGFP fusion protein expression by FACS analysis.

[0031] FIG. 8 is a depiction of a gene trapping of PGT5A-transfected PA317 cells. (A) PA317 cells transfected with pGT5A showed a 3.6% of hrGFP-positive cell population. (B) Sorting of the hrGFP-positive cell population in (A) by FACS cell sorter, hrGFP-positive population was enriched to 95% after 2 weeks of cell culture.

[0032] FIG. 9 is a depiction of gene expression of hrGFP in gene trapped PA317 cells. RT-PCR was performed on total RNA extracted from sorted cells in FIG. 7 and FIG. 8, and PCR product was electrophoresed in 2% agarose gel. The whole length of hrGFP transcripts driven by trapped cellular promoter (GT5A/PA317) were amplified by hrGFP specific primers after cDNA synthesis as indicated with an arrow. Transcripts from GT5Z in PA317 (GT5Z/PA317) and PA317 without vector (PA317) were used as a positive and negative control.

[0033] FIG. 10 is a depiction of gene trapping of GT5A vector in human lung cancer cells, A549, after viral transduction. (A) A549 cells without transduction analyzed

by FACS. (B) A549 cells with GT5A-transduction analyzed by FACS showed the hrGFP-positive population is 1.68% after gene trapping.

[0034] FIG. 11 is a depiction of gene trapping of GT5A vector in NIH3T3 cells. Mixed population of GT5A-trapped NIH3T3 cells were sorted and cultured for three weeks and then analyzed by FACS comparing to untransduced cells. Different intensities of hrGFP were shown in four different major groups.

[0035] FIG. 12 is a depiction of hrGFP gene expression of single-cell clones from GT5A-trapped NIH3T3 cells. Individual single-cells were sorted into 96-wells plate and cultured to a sufficient population for FACS analysis. A6P1 and C4P2, C8P2 and H8P2 were analyzed at two different events while compared to untransduced NIH3T3 cells.

[0036] FIG. 13A-D is a depiction of gene trapping with an  $\alpha$ 1,3-galactosyl transferase as a reporter gene in human melanoma cell line, A375. (13A) Schematic diagram of serial gene trapping vectors with  $\alpha$ 1,3-galactosyl transferase ( $\alpha$ 1,3-gal) gene.  $\alpha$ 1,3 galactosyl transferase is an enzyme that will generate galactosylated products only in the Golgi, by using its gene as a marker for gene trapping it will select for those gene trapping events that generate fusions between  $\alpha$ 1,3 galactosyl transferase and proteins that have a Golgi localization signals. LTR, long terminal repeat; SV40, simian virus type 40 early promoter; ZeoR, Zeocin resistant gene; CMV, CMV early promoter; NeoR; neomycin resistant gene; pA, bovine growth hormone poly-A signal. SA, human  $\gamma$ -globin intron 2 splicing acceptor; SD, synthetic splicing donor. pA, NeoR, CMV,  $\alpha$ 1,3 gal, SA or SD, ZeoR and SV40 are in anti-sense orientation against LTRs. (13B) Gene trapping of pGT7A in A375/AMIZ cells. Cells were labeled with lectin conjugated with FITC for FACS analysis. Lectin binds to  $\alpha$ 1,3 gal epitopes on cell surface to show successful gene-trapping. (13C) Gene trapping in A375/AMIZ cells 3 days post transfection of pGT7AH. (13D) Splicing function and functional  $\alpha$ -1,3  $\alpha$ -gal/ZeoR fusion protein were demonstrated by lectin/FITC-positive cells.

[0037] FIG. 14A – 14D show representative examples of cell sorting by FACS into different gates according to expression levels of the fluorescent reporter gene. In these examples, a normal (HMEC, FIGS. 14A and 14C) and a cancer (MCF7, Figs 14C and 14D) breast cell lines were transduced with retroviral vectors HSG (Figs 14C and

14D) or pGT-FS0 (Figs 14A and 14B). Cells were first enriched by a first round of cell sorting by FACS (second panel from the top in every FIG.) and then were subsequently sorted into 4 gates with different levels of expression of the fluorescent marker protein.

[0038] FIG.15 is a schematic depicting a vector of the invention which utilizes homologous recombination as the integration strategy. The repeat sequences are engineered to flank the assay marker gene and then introduced to the cell.

[0039] FIG. 16 is a diagram depicting the concept of frame alignment. Only 1 in 3 integrants will be in frame, based upon the triplet codon scheme so that only 1 in three integrated vectors will be functional and result in translation of the assay marker.

[0040] FIG. 17 is a schematic depicting the inverse PCR procedure for recovering genomic tags associated to vector or viral integration events. A method of cleaving said cellular DNA such that inserted DNA (with sequence known to the operator) is cleaved once and flanking cellular DNA of unknown sequence is cleaved again in the regions contiguous to the inserted piece of DNA. Cleavage of the DNA occurs in a fashion generating ends that permit the circularization of DNA fragments producing a molecule with the sequence known to the operator flanking both sides, and continuous with, a variable length of cellular DNA of unknown sequence. The region containing the unknown DNA is then amplified and sequenced.

[0041] FIG. 18A-B is a schematic depicting one of the possible experimental procedures to carry out the method denominated 5' Serial Analysis of Viral Integration (5'SAVI). After the SA joins to the splicing donor (SD) of the integrated cellular gene by cellular splicing mechanism, reverse transcription is employed to convert this hybrid RNA transcript into a complementary double stranded cDNA (cDNA) following standard methods for full length total cDNA synthesis. This cDNA is then subjected to restriction enzyme digestion with a Type IIS restriction enzyme which will cut the cDNA into the sequences corresponding to the cellular exon ten to twenty bases away from the SD/SA junction depending on which Type IIS restriction enzyme is used. A biotin-labeled primer #1 with a sequence specific for the marker gene is then employed to generate a ssDNA fragment that extends into the cellular exon fused to the marker gene. Collection of this biotin-ssDNA by streptavidin conjugated magnetic beads enriches these specific

ssDNA for subsequent DNA terminal transferase reaction. Poly-deoxynucleotide can be added onto these ssDNA as a tail at their 3' end. An oligonucleotide primer complementary to the polymer tail and a second primer #2 nested with respect to primer #1 on the marker gene can therefore be used to amplify by PCR this 3' end of the cellular exon fused to the 5' side of the marker exon. These short tags or amplification fragments from different integrated genes can, by ligation reactions, be made into longer DNA fragments that are subsequently sequenced.

**[0042]** FIG. 18C-D is a schematic depicting another possible experimental approach to carry out the 5' SAVI method. In this second version of the 5' SAVI method, a biotinylated primer #1 specific to the complementary sequence of the marker exon is used to prime cDNA synthesis by reverse transcriptase. Next, a polynucleotide tail is added to this single stranded cDNA by the enzyme terminal transferase. An oligonucleotide primer complementary to this homopolymeric tail is then used to drive the synthesis of the complementary second DNA strand. Double stranded DNA products are subjected to a type IIS restriction enzyme digestion and the digestion products are purified with magnetic streptavidin beads. An adaptor is ligated to the end generated by the type IIS restriction enzyme and the products are amplified by PCR with a primer corresponding to the adaptor sequence and with a primer #2 specific to the marker gene and nested with respect to primer #1. Amplification products are ligated together into high order polymeric structures, cloned into sequencing vectors and sequenced.

**[0043]** Figure 18 E-F depicts the method of 3' SAVI. In this case, the marker exon contains a type IIS restriction enzyme at the 3' end of the exon sequence immediately followed by a splice donor consensus sequence. After mRNA purification and double stranded cDNA synthesis and digestion with a type IIS restriction enzyme, a double stranded DNA adaptor can be ligated to the end generated by the type IIS restriction enzyme. Amplification of fragments containing marker sequences can be accomplished by PCR amplification using a primer #1 specific to the marker exon sequence and a second primer corresponding to the ligated adaptor. After PCR amplification, fragments of equal length can be cloned into high order polymeric structures, cloned into sequencing vectors and sequenced.

[0044] FIG. 18 G-H is a schematic of the Serial Analysis of Viral Integration (SAVI) method that permits the simultaneous identification of cellular exon boundaries. The method starts by isolating mRNA from the cell, and generating full length double stranded cDNA by reverse transcription. The cDNA is subjected to a Type IIS restriction enzyme (RE) that recognizes the first and second Type IIS RER sites, cleaving the cDNA upstream of the first Type IIS RER site and downstream of the second Type IIS RER site. Then, the fragment is self-ligated generating a circular molecule, where the sequence tags from the upstream and downstream cellular exons are fused in inverse orientation generating a di-tag. This di-tag is then amplified by inverse PCR using marker-specific primers. Following amplification, the fragments are subjected to digestion with the restriction enzymes that cut at the non-Type IIS REs, separation and ligated together to form a concatamer, which is then sequenced by appropriate methods.

[0045] FIG. 19 is a non-limiting flow diagram demonstrating the entire process. This FIG. delivers a rudimentary overview of the process of the invention. The process begins with two different populations of cells to be compared. Each population of cells to be compared will have been marked genetically by a vector containing marker/s-peptides to facilitate detection and determination of relative concentration of marker/s. Left portion of middle panel demonstrates separation of populations of cells based on relative amount of marker present in the tagged cells. Sequences flanking the vector will be determined by but not limited to SAVI method or an inverse PCR procedure for recovering genomic tags associated with vector or viral integration events. Valid tags will then be compared to public and commercial data bases and annotated into our own data bases. As can be seen at each stage alternatives exist for each step.

[0046] FIG. 20 is a diagram demonstrating the layers of information which may be assayed to identify the real state of cell (furthest outward circle). Those who assay DNA and raw sequence data determine gene function based on sequence similarity, gene structure, and evolutionary relationships. Missing from this data is any mRNA or translational modification data. Those who assay mRNA gain a prediction of a protein profile based on the assumption that protein levels are directly proportional to mRNA. An assumption which is proving to be erroneous. Closest of all these methods to the real cell

state is the method of the invention which detects actual cellular protein levels by direct measurement.

[0047] FIG. 21 is a depiction of a successful gene trapping in pGT5A-transfected PA317 cells. NcoI restriction site located at the 5' end of hrGFP marker gene and an EcoRI at the Oligo-dA primer were used as cloning sites for gene trapped sequence into a sequencing vector which was digested with NcoI and EcoRI. After BLAST searching against mouse EST database in GenBank, the sequence trapped by pGT5A demonstrates 99% homology to a high mobility group protein, HMGI-C, a nuclear phosphoprotein that contains three short DNA-binding domains (AT-hooks) and a highly acidic C-terminus.

[0048] FIG. 22 is a depiction of gene trapping of an exon with unknown biological function in pGT5A-transfected PA317 cells. NcoI restriction site located at the 5' end of hrGFP marker gene and an EcoRI at the oligo-dA primer were used as cloning sites for gene trapped sequence into a sequencing vector which was digested with NcoI and EcoRI. After BLAST searching against the EST database in GenBank, the sequence trapped by pGT5A is 95% match to a NCI\_CGAP\_Li9 Mus musculus cDNA clones, BF539247.1/BF533319.1/ which have been found in the cDNA libraries from Salivary gland and liver.

[0049] FIG. 23 Compilations of genes identified by this technology, classified according to their subcellular localization.

[0050] FIG. 24 Compilations of genes identified by this technology, classified according to their functional role.

[0051] FIG. 25A-B Illustrate the utilization of this invention to screen for cellular protein domains that interact with a target protein of interest. As shown in FIG. 25A, a stable cell line expressing a fusion between the target protein (bait protein) fused to one of the subunits of the reporter system is constructed. Gene trapping is performed on this stable cell line using retroviral vectors that encode the second subunit of the reporter system in an exon acceptor configuration. As shown in FIG. 25B, this generates a library of gene trapped protein domains fused to the second subunit of the reporter system. Upon interaction of the protein domains fused to each subunit of the reporter system, the reporter systems gains functionality allowing to generate a fluorescent signal either by

fluorescence resonance energy transfer (FRET, for CFP/YFP system) or by enzymatic conversion of a pro-fluorescent substrate ( $\alpha/\omega$  beta lactamase split system, PNAS 2002, 99:3469; Nat. Biotech. 2002, 20:619).

## **DETAILED DESCRIPTION OF THE INVENTION**

### **Definitions**

[0052] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxyl orientation, respectively. Numeric ranges are inclusive of the numbers defining the range and include each integer within the defined range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. Unless otherwise provided for, software, electrical, and electronics terms as used herein are as defined in The New IEEE Standard Dictionary of Electrical and Electronics Terms (5<sup>th</sup> edition, 1993). As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings and are more fully defined by reference to the specification as a whole:



**[0053]** By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Canteen, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA) See, e.g., Diagnostic Molecular Microbiology: Principles and Applications, D. H. Persing et al., Ed., American Society for Microbiology, Washington, D.C. (1993). The product of amplification is termed an amplicon.

**[0054]** The term "antibody" includes reference to antigen binding forms of antibodies (e.g., Fab, F(ab).sub.2). The term "antibody" frequently refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bi-specific antibodies).

**[0055]** The term "assay marker" or "reporter" refers to a gene product that can be detected in experimental assay protocol, such as marker enzymes, antigens, amino acid sequence markers, cellular phenotypic markers, nucleic acid sequence markers, and the like.

**[0056]** The term "assaying for the expression" of a protein coding sequence means any test or series of tests that permits cells expressing the protein to be distinguished from those that do not express the protein. Such tests include biochemical and biological tests and use either "selectable markers" or "assay markers."

**[0057]** As used herein, "chromosomal region" includes reference to a length of a chromosome that may be measured by reference to the linear segment of DNA that it comprises. The chromosomal region can be defined by reference to two unique DNA sequences, i.e., markers.

**[0058]** A "cloning vector" is a DNA molecule such as a plasmid, cosmid, or bacterial phage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a selectable marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Selectable marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

**[0059]** The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids which encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide of the present invention is implicit in each described polypeptide sequence and is within the scope of the present invention.

**[0060]** As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for its native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

**[0061]** The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W)

**[0062]** See also, Creighton (1984) *Proteins* W. H. Freeman and Company. The term "detectable marker" encompasses both the selectable markers and assay markers. The term "selectable markers" refers to a variety of gene products to which cells transformed with an expression construct can be selected or screened, including drug-resistance markers, antigenic markers useful in fluorescence-activated cell sorting, adherence markers such as receptors for adherence ligands allowing selective adherence, and the like.

**[0063]** By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as are present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum*, or the ciliate Macronucleus, may be used when the nucleic acid is expressed therein.

**[0064]** When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed.

**[0065]** An "expression vector" is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements including promoters, tissue specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

**[0066]** The term "expression system" is used herein to refer to a genetic sequence which includes a protein encoding region which is operably linked to all of the genetic signals necessary to achieve expression of the protein encoding region. Traditionally, the expression system will include a regulatory element such as a promoter or enhancer, to increase transcription and/or translation of the protein encoding region, or to provide control over expression. The regulatory element may be located upstream or downstream of the protein encoding region, or may be located at an intron (non coding portion) interrupting the protein encoding region. Alternatively it is also possible for the sequence of the protein encoding region itself to comprise regulatory ability.

**[0067]** The term "functional splice acceptor" refers to any individual functional splice acceptor or functional splice acceptor consensus sequence that permits the construct of the invention to be processed such that it is included in any mature, biologically active mRNA, provided that it is integrated in an active chromosomal locus

and transcribed as a contiguous part of the pre-messenger RNA of the chromosomal locus.

**[0068]** As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

**[0069]** The term "host cell" encompasses any cell which contains a vector and preferably supports the replication and/or expression of the vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. The term as used herein means any cell which may be in culture or in vivo as part of a unicellular organism, part of a multicellular organism, or a fused or engineered cell culture.

**[0070]** The term "internal ribosome entry site" (IRES) is an element which permits attachment of a downstream coding region or open reading frame with a cytoplasmic polysomal ribosome for purposes of initiating translation thereof in the absence of any internal promoters. An IRES is included to initiate translation of selectable marker protein coding sequences. Examples of suitable IRESes that can be used include the mammalian IRES of the immunoglobulin heavy-chain-binding protein (BiP). Other suitable IRESes are those from the picornaviruses. For example, such IRESes include those from encephalomyocarditis virus (preferably nucleotide numbers 163-746), poliovirus (preferably nucleotide numbers 28-640) and foot and mouth disease virus (preferably nucleotide numbers 369-804). Thus, the viruses are located in the long 5' untranslated regions of the picornaviruses which can be removed from their viral setting in length to unrelated genes to produce polycistronic mRNAs.

**[0071]** The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the

incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

**[0072]** The term "isolated" refers to material, such as a nucleic acid or a protein, which is: (1) substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment; or (2) if the material is in its natural environment, the material has been synthetically (non-naturally) altered by deliberate human intervention to a composition and/or placed at a location in the cell (e.g., genome or subcellular organelle) not native to a material found in that environment. The alteration to yield the synthetic material can be performed on the material within or removed from its natural state. For example, a naturally occurring nucleic acid becomes an isolated nucleic acid if it is altered, or if it is transcribed from DNA which has been altered, by means of human intervention performed within the cell from which it originates. See, e.g., Compounds and Methods for Site Directed Mutagenesis in Eukaryotic Cells, Kmiec, U.S. Pat. No. 5,565,350; In Vivo Homologous Sequence Targeting in Eukaryotic Cells; Zarling et al., PCT/US93/03868. Likewise, a naturally occurring nucleic acid (e.g., a promoter) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome not native to that nucleic acid. Nucleic acids which are "isolated" as defined herein are also referred to as "heterologous" nucleic acids.

**[0073]** As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

**[0074]** As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second

sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

**[0075]** The term "polymerase chain reaction" or "PCR" refers to a procedure described in U.S. Pat. No. 4,683,195, the disclosure of which is incorporated herein by reference.

**[0076]** As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons as "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells.

**[0077]** The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids.

The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation,  $\gamma$ -carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. It will be appreciated, as is well known and as noted above, that polypeptides are not entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well.

[0078] The term "primer" refers to a nucleic acid which, when hybridized to a strand of DNA, is capable of initiating the synthesis of an extension product in the presence of a suitable polymerization agent. The primer preferably is sufficiently long to hybridize uniquely to a specific region of the DNA strand.

[0079] As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

[0080] The term "promoterless" refers to a protein coding sequence contained in a vector, retrovirus, adenovirus, adeno-associated virus or retroviral provirus that is not directly or significantly under the control of a promoter within the vector, whether it be in RNA or DNA form. The vector, plasmid, viral or otherwise, may contain a promoter, but that promoter cannot be positioned or configured such that it directly or significantly regulates the expression of the promoterless protein coding sequence.

[0081] The term "protein coding sequence" means a nucleotide sequence encoding a polypeptide gene which can be used to distinguish cells expressing the polypeptide gene from those not expressing the polypeptide gene. Protein coding sequences include those commonly referred to as selectable markers. Examples of protein coding sequences include those coding a cell surface antigen and those encoding enzymes. A representative list of protein coding sequences include thymidine kinase,  $\beta$ -galactosidase, tryptophan synthetase, neomycin phosphotransferase, histidinol dehydrogenase, luciferase, chloramphenicol acetyltransferase, dihydrofolate reductase



(DHFR); hypoxanthine guanine phosphoribosyl transferase (HGPRT), CD4, CD8 and hygromycin phosphotransferase (HYGRO).

**[0082]** As used herein "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under-expressed or not expressed at all as a result of deliberate human intervention. The term "recombinant" as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

**[0083]** As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a host cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

**[0084]** A "recombinant host" may be any prokaryotic or eukaryotic cell that contains either a cloning vector or an expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the clone genes in the chromosome or genome of the host cell.

**[0085]** The terms "recombinant virus vector" refers to any recombinant ribonucleic acid molecule having a nucleotide sequence homologous or complementary with a nucleotide sequence in an RNA virus that replicates through a DNA intermediate, has a virion RNA and utilizes reverse transcriptase for propagation of virus in a host cell. Such viruses can include those that require the presence of other viruses, such as helper viruses, to be passaged. Thus, retroviral vectors or retroviruses are intended to include those containing substantial deletions or mutations in their RNA.

**[0086]** The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

**[0087]** The terms "tag" or "tagged" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g.,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (or reporter genes) (e.g., horseradish peroxidase, .beta.-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

**[0088]** The term "translational stop sequence" refers to a sequence that code for the translational stop codons in three different reading frames. This translational stop sequence is physically located downstream (3') of the splice acceptor sequence, but upstream (5') of the selectable marker fusion protein translation initiation site. It causes truncation of the peptide chain encoded by exons upstream of the retroviral vector at the chromosomal locus. It also prevents the translational reading frame of the genomic locus from proceeding into the selectable marker gene of the invention, thus preventing potential translation of it in a non-sense reading frame.

**[0089]** As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

**[0090]** The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

**[0091]** (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

**[0092]** (b) As used herein, "comparison window" includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence, a gap penalty is typically introduced and is subtracted from the number of matches.

**[0093]** Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif.; GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software

Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73:237-244 (1988); Higgins and Sharp, *CABIOS* 5:151-153 (1989); Corpet, et al., *Nucleic Acids Research* 16:10881-90 (1988); Huang, et al., *Computer Applications in the Biosciences* 8:155-65 (1992), and Pearson, et al., *Methods in Molecular Biology* 24:307-331 (1994). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

**[0094]** Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters. Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997). Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology-Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue

alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915).

**[0095]** In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, Comput. Chem., 17:149-163 (1993)) and XNU (Claverie and States, Comput. Chem., 17:191-201 (1993)) low-complexity filters can be employed alone or in combination.

**[0096]** (c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such

conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif., USA).

[0097] (d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0098] (e) (i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, or preferably at least 70%, 80%, 90%, and most preferably at least 95%.

**[0100]** Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. However, nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

**[0101]** (e) (ii) The terms "substantial Identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, ore preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Optionally, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970). an indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes.

**[0102]** The terms "oligoclonal", "polyclonal" applied to cell populations indicates a population of cells where some cells within that population are not genetically identical to the rest of the cells of that population. Conversely, the term "monoclonal" or "monoclonal cell population" indicates that all cells within that population are genetically identical. Differences in the "genetic identity" of a population of cells in the context of this invention arise by random retroviral integration into different genomic insertion sites.

**[0103]** The invention relates to a method for identifying a particular protein profile for a cell that can be used for diagnosis or as a target site for a drug. According to the invention activity at specific genetic loci is correlated with the functional state and/or

concentration of product proteins in a test cell and is then compared to a reference cell for elucidation of differential and quantitative protein expression profiles.

**[0104]** The method can be used to identify gene or gene products associated with a biological process or state of interest, to identify proteins including entire pathways of expression associated with a particular state, to screen cells for the identification of a particular protein profile that is associated with said state for diagnosis, to identify novel proteins associated with particular cell types or states, or even to identify polymorphisms in genes causing differential proteins between genes. This can include the assay for expression or not of a particular protein or a relative quantitative expression profile.

**[0105]** The method employs three basic steps to achieve its objective. First the test cell is transformed with a promoterless polynucleotide construct to "tag" the cell by integration of a detectable marker or reporter nucleotide sequence in the genome of the cell. The detectable marker sequence encodes a protein that is only produced when the integration event has occurred in a cellular gene in such a fashion that the marker protein is produced under the transcriptional control of a cellular gene promoter, resulting in an interrupted gene product and preferably a fusion protein incorporating the tag.

**[0106]** This is achieved by inclusion of the marker nucleotide sequence in a polynucleotide construct, typically a vector, with no promoter operably linked to the marker nucleotide sequence. Thus expression of the marker is dependent upon initiation of transcription from the target cell genome. Any vector can be used according to the invention which is capable of integrating into the genome of said target cell, this can include but is not limited to, e.g., parvoviruses, foamy viruses, retrotransposons, etc., and/or naked DNA). In a preferred embodiment the vector is a defective retrovirus, packaging of the defective retroviral genome and insertion of the defective retroviral genome via abortive infection into the DNA of the cells to be analyzed.

**[0107]** Production of the marker indicates that the construct has been integrated into an actively transcribed region of the cellular genome and production/accumulation of the marker protein becomes dependent upon transcription initiated at cellular promoters. Performance of the foregoing aspect of the instant invention results in a mixed population of cells wherein the marker is integrated into a different gene in each cell. In some



embodiments of the instant invention, this initial mixture of the cells may be separated into monoclonal populations containing genetically identical cells in which the marker is integrated into the same gene. These monoclonal populations can be obtained by a number of different methods. For example, the original mixture of cells with the integrated marker can be seeded at a very low concentration, which allows each individual cell of the mixture to expand into a monoclonal.

**[0108]** The advantage of isolating individual clones is that these clones can be used, for example, as an assay system for screening of small molecule drugs that block the expression or function of the fusion protein tagged with the marker exon. This screening is based on measuring the variations in fluorescence signal produced by different drugs at different concentrations.

**[0109]** In the second step of the invention, cells containing integrated sequences can be sorted and fractionated on the basis of the expression of the marker protein. Again any of a number of different sorting methodologies can be used depending on the chemical physical or mechanical characteristics of the marker gene. In a preferred embodiment the marker is a fluorescent protein and quantitation of protein is performed by Fluorescence Activated Cell Sorting (FACS) such that in addition to quantifying protein, cells expressing given levels of protein may be sorted and collected in fractions (there may be any number of fractions, e.g. 5, 10, 20, 25, 50, 100, etc., depending upon the desired level of resolution). Also, as mentioned above, the tagged population of cells can be sorted into individual clones, which would allow measuring the level of expression of each tagged protein in all possible stages of the cell cycle, and would also provide a direct link between the expression level of the fusion protein and the identity of the trapped gene.

**[0110]** In addition other means such as the use of ferrous metal conjugates and electromagnetic force can be used for expression-dependent fractionation of cells. The FACS sorting can be employed to isolate single cells; each of these cells may then be used to establish an independent clone of homogeneous cells in which the marker is integrated into one particular gene.

[0111] The high speed and resolution of the invention allow for the first time, real time analysis of molecular pathways of activation including but not limited to signal transduction via phosphorylation levels of targets (*i.e.*, direct measurements of phosphatases/kinases and/or production of cyclic AMP). The invention also provides analysis of gated and non gated channels to monitor signaling via  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ , pH and other trace elements. This analysis can also monitor protein-protein interactions as well using fluorescent Ab, fluorescent Ag, fluorescent ligand, fluorescent receptor, fluorescent substrates or non-fluorescent substrates that become fluorescent after enzymatic cleavage/activation or even conventional colorimetric enzyme-substrate based reactions. The combination of speed and precision offered by FACS has not been fully demonstrated using other methods of analysis; however, other methods may be used. In another embodiment electromagnetic forces can also be used to separate and quantitate cells by the expression of the marker gene product.

[0112] In the final step, once sorted into oligoclonal or monoclonal subpopulations (based upon the level of marker peptide expression), DNA, RNA, and/or protein are isolated from the cells in each subpopulation/monoclonal and analyzed. This analysis includes determination of the cellular DNA sequences into which the marker DNA has been inserted. Then comparison is made against a reference cell to identify differential protein expression that is correlated with a particular state, for example a disease state such as cancer for use as a diagnostic or to identify a potential target for drug intervention.

[0113] Rather than isolating and amplifying each individual cell which has acquired an integrated and expressed marker tag and then subsequently analyzing the site of insertion and the level of protein expression, estimates of these values may be obtained by analyzing statistically significant numbers of cells with such integrants that have been clustered together by virtue of demonstrating an approximately equivalent level of gene expression.

[0114] For example, if in a population of 1,000,000 cells, 10,000 cells with integration events analyzing a series of different genes in a population demonstrate a mean marker peptide concentration of value x, where x represents the mean marker

peptide concentration seen in the first percentile of cells detected to have any expression of the marker peptide. If integration sites are determined in any (or all) of these cells, the genes where they have integrated are said to be expressed in the lowest percentile of detectable expression. This can be said independent of any knowledge that relates a specific integration event in a specific gene in a specific clone of cells to a specific protein level. By applying appropriate statistical methods and examining a large enough number of integration events, the need to obtain and analyze specific clones is obviated for the purpose of determining relative levels of protein expression from a given genetic locus. This initial cluster analysis of a subpopulation may be followed up by analysis of individual genes from monoclonal populations of cells isolated as described above.

**[0115]** In further embodiments the differential expression data can be used with statistical methods to assign marker peptide expression levels for each interrupted gene. In further yet embodiments a database which incorporates this newly generated data, with other data sources, is combined to produce a record on the relationship of gene expression (at the RNA and protein level) to the function of the cell can be generated. In cases where the cells under study can be obtained in both cancerous and normal conditions, comparisons of the relative gene expression can be used to identify genes which can serve either as diagnostic markers of pathology or as sites of pharmacologic intervention for treatment of cancer. Similarly, other diseases can be analyzed merely by substituting the source of cells for analysis.

**[0116]** Each of the foregoing steps will now be described in more detail below. It is understood that for each step numerous expedients may be employed as well as alternative molecular biology techniques currently or yet to become available which achieve the same results. Choice of reaction agents, protocols etc is considered nothing more than routine optimization of experimental parameters based upon the teaching herein and are intended to be within the scope of the invention. FIG. 19 is a flow diagram depicting an overview of the process including several specific examples of alternatives available for each step.

### **Identification and Selection of Test and Reference Cell Types**

**[0117]** According to the invention a comprehensive protein profile is generated from any cell type of interest. A test cell can be any cell, or portion thereof with genetic material. A reference cell can be any cell type the difference in protein expression patterns and levels is desired to be measured against. Preferably the cells are maintained as similar to their native state as possible and culture techniques, incubation times etc. are performed identically between the two to minimize any non-naturally occurring differences. For example, development of the comprehensive protein profiles of pre-cancerous, and malignant test cells and a normal reference cell could be achieved according to the invention. Such identifiers of protein signatures will characterize molecular events of tumor development and cellular mechanisms involved.

**[0118]** Recent initiatives in identification of molecular fingerprints of tumors have been focused on studies of DNA and mRNA levels. These studies indicate that gene expression paths in two tumor samples from the same individual were almost always more similar to each other than either was to any other sample and that tumors could be classified in subtypes distinguished by differences in their gene expression patterns.

**[0119]** According to the invention, a test cell and a reference cell could be obtained from the same patient to get an individual protein fingerprint that can be used to diagnose or treat that patient. For example when a tumor is excised, often a margin of non transformed cells is removed as well. Protein profiling can help to ensure that the cells removed all had similar profiles to normal cells rather than the metastatic cells from the same patient.

**[0120]** Comparisons may be made according to the invention from different cancers (e.g. lung, breast, colon, melanoma), different stages of malignant progression from corresponding normal tissue to highly malignant primary site and/or metastatic site, tumors caused by endemic/local agents (e.g. environmental agents (asbestos, infectious agents), tissues surrounding the incipient tumor (e.g. blood cells), extracts from body fluids (e.g. cancer cells of the urinary tract may be shed into urine), and tumors from species other than human.

**[0121]** One example of cell lines that may be used as test cells include human tumor cell lines. For example human tumor cell lines representing a broad spectrum of human tumors and exhibiting acceptable properties and growth characteristics may be grown according to standard operating procedure for cell line expansion, cryopreservation and characterization. Examples of human cancer cell lines which may be used according to the invention include: Lung Cancer Human Cell Lines (Non-small cell lung cancer adenocarcinoma cell line, A549; adenosquamous cell carcinoma, NCI-H125; squamous cell carcinoma, SK-MES-1, bronchial-alveolar carcinoma, NCI-M322; large cell Carcinoma, A 427, mucoepidermoid carcinoma, NCIM292, small cell lung cancer (SCLC) "Classic", NCI-M69; SCLC "Variant", NCI-M82; SCLC "Adherent", SHP77; colon cancer human cell lines (COLO 205, DLD-1, HCT-15, HT29, LoVo); breast cancer human cell lines, (MCF7 WT, MCF7 ADR, MDA-MB-231, HS 578T); prostate cancer human cell lines (D4 145, LNCaP, PC-3, UMSCP-1); melanoma human cell lines (RPMI-7951, LOX, SK-MEL 2, SK-MEL-5, A 375); renal cancer human cell lines (A 498, A 704, Caki-1, SNI2 C, UO-31); ovarian cancer human cell lines (IGROV-1, OVCAR-3, SK-OV-3, A2780, OVCAR-4, OVCAR-5, OVCAR-8); leukemia human cell lines (Molt-4, RPMI 8336, P388, P388/ADR-Resist CCRF-CEM, CCRF-SB); central nervous system cancer human cell lines (SF 126, SF 295, SNB19, SNB 44, SNB 56, TE 671, 4251); sarcoma human cell lines (A-204, A 673, MS 913T, Ht 1080, Te 85); head and neck squamous cancer human cell lines (UM-SCC-MB,C, UM-SCC-21A, UM-SCC-22B); normal fibroblasts (MRC-5-lung, human, CCD-194Lu-lung, human, IMR-90-lung, human, NIH 3T3-mouse, embryo).

**[0122]** Another example of cell types which could be used includes primary cells derived from normal or cancer tissue specimens such as a tissue specimen obtained from normal and/or cancerous tissue that is disaggregated using dissociating enzymes and single cell suspension that is enriched, purified and characterized using MACS tumor cell reagents.

**[0123]** In yet another embodiment test and reference cells can be used to develop protein profiles associated with aging such as different stages of ontogenesis, for example protein profiles of embryonic liver-derived hematopoietic stem cell (HSC) vs. cord blood HSC vs. young adult HSC vs. old age organism-derived HSC.

[0124] In yet another embodiment protein profiles of cells from Neurodegenerative diseases which as patients with Alzheimer's disease, Parkinson's disease may be assayed.

[0125] In yet another embodiment profiles may be obtained for other age-related conditions such as male pattern baldness.

[0126] In yet another embodiment protein profiles can be obtained from human pathological conditions such as genetic diseases (inborn errors of metabolism: Adenosine deaminase deficiency, cystic fibrosis, Duchenne's muscular dystrophy).

[0127] In yet another embodiment protein profiles may be obtained for multifactorial and somatic genetic diseases (hypertension, coronary artery disease, obesity, and diabetes mellitus).

[0128] In yet still another embodiment profiles may be obtained for other non-genetic diseases (AIDS and other infectious diseases).

[0129] In yet still another embodiment profiles may be obtained for autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, etc.).

[0130] In yet another embodiment profiles may be obtained for human non-disease traits such as physical traits (athletic abilities, visual acuity); cognitive and personality traits (musical ability, cognition, memory, male-pattern-baldness).

[0131] In yet another embodiment two cells of the same type may be assayed to identify alternative gene forms, such as polymorphic loci etc.

[0132] Further, as can be seen, any cell type can be used according to the invention including but not limited to, microorganisms, plants, invertebrates, vertebrates, and mammals.

#### **Integration of Assay Marker Peptide-encoding Sequences into the Genetic Material of Test and Reference Cells.**

[0133] According to the invention the process begins by the insertion of an assay marker DNA sequence into the genome of a test cell to be analyzed. This assay marker sequence includes any expressed molecule which can be screened in a defined assay

system such that the cells may be identified, selected, sorted and/or preferably quantified, based upon the expression of the marker sequence. In a preferred embodiment this marker sequence or (tag) will be a chromophore which will fluoresce (such as humanized renilla green fluorescence protein). Other examples of assay marker sequences which may be used according to the invention include  $\alpha$ -1-3 galactosyltransferase, sodium/iodine symporter, (or viral envelope protein could be used). Still other marker systems include but are not limited to any detectable cell surface displayed protein; other markers can be used such as lipid, lipoprotein, glycolipid, and glycoprotein targets that can be tagged with specific fluorescent compounds using labeled antibodies, direct chemical linkage and/or combination of direct and indirect tagging.

**[0134]** The marker peptide/fusion protein may be intracellular and dispersed throughout the cytoplasm or localized to specific intracellular compartments by leader sequences present on the marker peptide/fusion proteins(s). The marker peptide(s) can be incorporated into a single protein or into macromolecular complexes in which several different proteins (derived from multiple cellular genes) are linked by specific molecular interactions that demonstrate a unique fluorescent profile.

**[0135]** The marker peptide is only produced when the integration event has occurred in a cellular gene in such a fashion that the marker protein is produced under the transcriptional control of a cellular gene promoter. This is achieved by inclusion of the marker DNA sequence within a promoterless expression construct.

**[0136]** In a preferred embodiment the expression construct is included within an appropriate gene transfer vehicle which is then used to transduce cells to express the marker gene by the recipient host test cells. The gene delivery vehicle can be any delivery vehicle known in the art and can include simply naked DNA which is facilitated by a receptor mediated transfection or via homologous recombination. (see FIG. 15). In a homologous recombination embodiment a vector is engineered to have highly repeated sequences such as Alu flanking the assay marker gene so that recombination is facilitated at the repetitive sites causing integration of the nucleotide. Any of a number of vectors can be used, such vectors include but are not limited to eukaryotic vectors, prokaryotic vectors (such as for example bacterial vectors) and viral vectors including but not limited

to retroviral vectors, adenoviral vectors, adeno-associated viral vectors, lentivirus vectors (human and other including porcine), or any other vector which will stably integrate into the host cell genome.

[0137] The preferred embodiment of the invention will use vectors (DNA, RNA, DNA/RNA hybrids etc.) that contain markers which may be sorted to include but not limited to cell surface displayed or cytoplasmic protein; lipid, lipoprotein, glycolipid, and glycoprotein targets that can be tagged with specific fluorescent, chemiluminescent, or bioluminescent compounds using labeled antibodies, direct chemical linkage and/or combination of direct and indirect tagging. These vectors (see FIG. 2A-K, 13A) use either the processes of illegitimate recombination, homologous recombination, and/or viral vectors to integrate said markers into the genomic DNA of target cells (the integrated vector serves as a molecular bar code). Alu sequences are approximately 300 bp in length and are found on average every 3000 bp in the human genome. Alu or other highly repetitive sequences can be used to induce homologous recombination for insertion of the marker gene. The vectors will be delivered to the target cells via standard gene delivery methods to include but not limited to lipid mediated transfection (cationic, anionic, and neutral charged), activated dendrimers (PolyFect™ Reagent, SuperFect™ Reagent {Qiagen}), Phenylethyleneimide (PEI), receptor mediated transfection (fusogenic peptide/protein), calcium phosphate transfection, electroporation, particle bombardment, direct injection of naked-DNA, diethylaminoethyl (DEAE-dextran transfection) etc. Though the preferred embodiment is the use of plasmid based vectors, the use of other high efficiency viral vectors is not precluded.

[0138] The expression vehicles (vectors) of the invention can be engineered by any of a number of techniques known to those of skill in the art. The following is a summary of techniques for construction and transformation of the vectors of the invention.

#### **Genetic Engineering Techniques for Construction and Delivery of Vectors**

[0139] In a preferred embodiment the expression vehicles or vectors of the invention comprising the expression system also comprise a selectable marker gene to select for transformants as well as a method for selecting those transformants for



propagation of the construct in bacteria. Such selectable marker may contain an antibiotic resistance gene, such as those that confer resistance to ampicillin, kanamycin, tetracycline, or streptomycin and the like. These can include genes from prokaryotic or eukaryotic cells such as dihydrofolate reductase or multi-drug resistance I gene, hygromycin B resistance that provide for positive selection. Any type of positive selector marker can be used such as neomycin or Zeocin and these types of selectors are generally known in the art. Several procedures for insertion and deletion of genes are known to those of skill in the art and are disclosed. For example in Maniatis, "Molecular Cloning", Cold Spring Harbor Press. See also Post et al., Cell, Vol. 24:555-565 (1981). An entire transcription unit must be provided for the selectable marker genes (promoter-gene-polyA) and the genes must be flanked on one end or the other with promoter regulatory region and on the other with transcription termination signal (polyadenylation cite). Any known promoter/transcription termination combination can be used with the selectable marker genes. Examples of such systems include  $\beta$ -lactamase (penicillinase) and lactose promoter systems, (Chang et al., Nature, 1977, 198:1056); the Tryptophan (trp) promoter system (Goeddel, et al., Nucleic Acid Res., 1980, 8:4057) and the lambda derived P<sub>L</sub> promoter and N-gene ribosome binding site (Shimatake et al., Nature 1981, 292:128). Other promoters such as cytomegalovirus promoter or Rous Sarcoma Virus can be used in combination with various ribosome elements such as SV40 poly A. The promoter can be any promoter known in the art including constitutive, (supra) inducible, (tetracycline-controlled transactivator (tTA)-responsive promoter (tet system, Paulus, W. et al., "Self-Contained, Tetracycline-Regulated Retroviral Vector System for Gene Delivery to Mammalian Cells", J of Virology, January 1996, Vol. 70, No. 1, pp. 62-67)), or tissue specific, (such as those cited in Costa, et. Al., European journal of Biochemistry, 258 "Transcriptional Regulation of the Tissue-Type Plasminogen Activator Gene in Human Endothelial Cells: Identification of Nuclear Factors That Recognize Functional Elements in the Tissue-Type Plasminogen Activator Gene Promoter" pgs, 123-131 (1998); Fleischmann, M., et al., FEBS Letters 440 "Cardiac Specific Expression Of The Green Fluorescent Protein During Early Murine Embryonic Development" pgs. 370-376, (1998); Fassati, Ariberto, et al., Human Gene Therapy, (9:2459-2468) "Insertion Of Two Independent Enhancers In The Long Terminal Repeat Of A Self Inactivating Vector

Results In High-Titer Retroviral Vectors With Tissue Specific Expression" (1998); Valerie, Jerome, et. Al. Human Gene Therapy 9:2653-2659, "Tissue Specific Cell Cycle Regulated Chimeric Transcription Factors for the Targeting Of Gene Expression to Tumor Cells, (1998); Takehito, Igarashi, et. Al., Human Gene Therapy 9:2691-2698, "A Novel Strategy of Cell Targeting Based on Tissue-Specific Expression of the Ecotropic Retrovirus Receptor Gene", 1998; Lidberg, Ulf et.al. The Journal of Biological Chemistry 273, No.47, "Transcriptional Regulation of the Human Carboxyl Ester Lipase Gene In Exocrine Pancreas" 1998; Yu, Geng-Sheng et. Al., the Journal of Biological Chemistry 273 No. 49, "Co-Regulation of Tissue-Specific Alternative Human Carnitine Palmitoyltransferase IB Gene Promoters by Fatty Acid Enzyme Substrate" (1998)). These types of sequences are well known in the art and are commercially available through several sources, ATCC, Pharmacia, Invitrogen, Stratagene, and Promega. Alternatively, the marker delivery vector may not contain an independent transcription unit encoding a selectable marker, and selection in this case can be made solely based on the biological and phenotypic properties of the assay marker gene used for exon trapping.

**[0140]** The assay marker gene to be expressed can then be introduced into the vector of the invention. The foreign marker gene DNA typically will comprise a promoterless transcription unit.

**[0141]** In a most preferred embodiment the vector comprises a specifically engineered multi-cloning site within which several unique restriction sites are created. Restriction enzymes and their cleavage sites are well known to those of skill in the art.

**[0142]** In a preferred embodiment, a packaging cell line is transduced with a viral vector containing the marker nucleotide sequence to form a producer cell line including the viral vector. The producer cells may then be directly administered, whereby the producer cells generate viral particles capable of transducing the recipient cells.

**[0143]** In a preferred embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus,

human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus.

[0144] Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the therapeutic gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

[0145] The marker gene may be incorporated into the proviral backbone in several general ways. The most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter. However, in the context of the instant invention, a promoterless marker gene has to be introduced within the vector backbone, preferably in an orientation which is inverted with respect to that of the viral transcription so the splicing signals carried by the marker exon do not interfere with the vector splicing signals.

[0146] Efforts have been directed at minimizing the viral component of the viral backbone, largely in an effort to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

[0147] In one embodiment, the retroviral vector may be one of a series of vectors described in Bender, et al., J. Virol. 61:1639-1649 (1987), based on the N2 vector (Armentano, et al., J. Virol., 61:1647-1650) containing a series of deletions and

substitutions to reduce to an absolute minimum the homology between the vector and packaging systems. These changes have also reduced the likelihood that viral proteins would be expressed. In the first of these vectors, LNL-XHC, there was altered, by site-directed mutagenesis, the natural ATG start codon of gag to TAG, thereby eliminating unintended protein synthesis from that point.

[0148] In Moloney murine leukemia virus (MoMuLV), 5' to the authentic gag start, an open reading frame exists which permits expression of another glycosylated protein (<sup>ppr80</sup>gag). Moloney murine sarcoma virus (MoMuSV) has alterations in this 5' region, including a frameshift and loss of glycosylation sites, which obviate potential expression of the amino terminus of <sup>ppr80</sup>gag. Therefore, the vector LNL6 was made, which incorporated both the altered ATG of LNL-XHC and the 5' portion of MoMuSV. The 5' structure of the LN vector series thus eliminates the possibility of expression of retroviral reading frames, with the subsequent production of viral antigens in genetically transduced target cells. In a final alteration to reduce overlap with packaging-defective helper virus, Miller has eliminated extra env sequences immediately preceding the 3' LTR in the LN vector (Miller, et al., *Biotechniques*, 7:980-990, 1989).

[0149] The paramount need that must be satisfied by any gene transfer system for its application to gene therapy is safety. Safety is derived from the combination of vector genome structure together with the packaging system that is utilized for production of the infectious vector. Miller, et al. have developed the combination of the pPAM3 plasmid (the packaging-defective helper genome) for expression of retroviral structural proteins together with the LN vector series to make a vector packaging system where the generation of recombinant wild-type retrovirus is reduced to a minimum through the elimination of nearly all sites of recombination between the vector genome and the packaging-defective helper genome (i.e. LN with pPAM3).

[0150] In one embodiment, the retroviral vector may be a Moloney Murine Leukemia Virus of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al. (1987) and Miller, et al. (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon

has been deleted or altered such that the gag protein or fragment or truncations thereof, are not expressed.

**[0151]** In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral vector includes each of these cloning sites.

**[0152]** When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

**[0153]** The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

**[0154]** The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

**[0155]** The vector then is employed to transduce a packaging cell line to form a producer cell line. Examples of packaging cells which may be transfected include, but are not limited to the PE501, PA317, PSI-2, PSI-AM, PA12, T19-14X, VT-19-17-H2, PSI-CRE, PSI-CRIP, GP+E-86, GP+envAM12, and DAN cell lines. The vector containing the therapeutic nucleic acid sequence may transduce the packaging cells through any

means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and calcium phosphate co-precipitation. The producer cells then are administered directly to or adjacent to desired recipient cells.

**[0156]** In another embodiment, the retroviral vectors can be based on human immunodeficiency virus Type I, using backbones for vector and helper packaging plasmids as described by Naldini et al., *Science* 1996, 272: 263-267; Zufferey et al., *Nature Biotechnology* 1997, 15: 871-875; and Reiser et al., *Proc. Natl. Acad. Sci. USA* 1996, 93: 15266-15271. Moreover, these vectors can withstand a deletion in the 3' U3 region of the 3' LTR that turns them into self-inactivating vectors after integration into the target genome, without a negative impact in vector titers (Zufferey et al., *J of Virology* 1998, 72: 9873-9880; Miyoshi et al., *J. of Virology* 1998, 72: 8150-8157). In the context of the present invention, the self-inactivating modification would avoid transcription of RNA from the viral 5'LTR that would generate an antisense RNA to the cellular gene being trapped by the assay marker gene delivered by the vector.

**[0157]** Integration occurs within the transcribed region of a cellular gene in a fashion that renders the production/accumulation of the marker protein dependent upon transcription initiated at cellular promoters.

**[0158]** In yet another preferred embodiment the polynucleotide vector may include a "splice acceptor site" so that if the vector integrates in the proper orientation within an intron encoding region of a cellular gene, the marker protein is produced as a fusion product with a portion of whatever cellular protein is encoded by the gene where the insertion event has occurred (Inclusive but not limited to, e.g., inclusion of an internal ribosome entry site (IRES) prior to the start codon of the marker gene ensures it will be expressed whenever RNA from the cellular gene (where integration has occurred) is transported to the cytoplasm in a form that is translatable). (FIG. 1)

**[0159]** Similarly, multiple markers may be included such that one marker protein may be expressed as a fusion and a second marker protein may be expressed from an IRES (FIG. 2C). Constructions are also possible to acquire different pieces of information about integration sites, depending upon the positioning of splice acceptor and donor sites.

**[0160]** According to the invention serial gene-trapping vectors for the acquisition of the data needed to assign integration sites to specific genes and to mean marker protein expression levels. Examples of such vectors are shown on FIG. 2A-2K.

**[0161]** As shown in FIG. 15 the issue of frameshift is also important to consider as only 1 in 3 integrants will be functional. Due to the triplet organization of translation 2 out of 3 integrations will not result in functional assay marker production as they will result in frame shifts which will disrupt the translation of the marker gene despite its integration into an active region of the cellular genome. Thus in yet another preferred embodiment of the invention, a plurality of vectors are constructed which are only one base or two bases different from the start site of the marker gene. This will help to trap some exons which are not in frame after integration of the marker nucleotide.

**[0162]** The production of these various vectors is readily achieved by those skilled in the art. One methodology which may be used for creating the vectors is as follows. The vectors to be produced as defective retroviruses are transfected into a vector packaging cell lines containing a helper virus (inclusive of, but not limited to, retroviral AMIZ helper virus, or other retroelements (Young, W. B. and C. J. Link, Jr., Chimeric retroviral helper virus and picornavirus IRES sequence To eliminate DNA methylation for improved retroviral packaging cells. J. Virol, 2000. 74(11): p. 5242-9) which can prevent the unwanted silencing of helper virus by cellular DNA methylation (Young, W. B., G. L. Lindberg, and C. J. Link, Jr., DNA methylation of helper virus increases genetic instability of retroviral vector producer cells. J Virol, 2000. 74(7): p. 3177-87). This AMIZ helper virus-packaging cell line can produce vector titer up to  $2 \times 10^7$  CFU (colony formation unit)/ml.

**[0163]** In certain circumstances where the production of retrovirus is limited alternative methods of retroviral production can be performed using a chimeric adenovirus system to produce vector titers up to  $5 \times 10^9$  cfu/ml (Ramsey et al., Caplen et al.).

### **Sorting of Cells Based Upon Levels of Marker Peptide Expression**

[0164] Cells which express the marker are then sorted and preferably quantified by their level of expression to generate an expression profile for a particular cell type. Sorting or separation of the cells can be by any method which provides for the separation and preferably quantification based upon expression of the marker sequence. This could be by fluorescence activation sorting, mechanical sorting, charge or density magnetic or other methods.

[0165] A preferred method of sorting includes the use of flow cytometry. Flow cytometry seeks to utilize complex integration of optic, fluidic, and electronic components to develop fluorescence activated cell sorters (FACS) capable of rapid interrogation of cells containing useful fluorescent marker/s in real time.

[0166] Marker which may be sorted by this method include cell surface displayed protein; lipid, lipoprotein, glycolipid, and glycoprotein targets that can be tagged with specific fluorescent compounds using labeled antibodies, direct chemical linkage and/or combination of direct and indirect tagging.

[0167] One alternative embodiment includes the use of high-sensitivity/high-density plate readers to detect chemiluminescent signals (range  $1 \times 10^{-18}$  M to  $1 \times 10^{-21}$  M) or with concomitant decreased sensitivity conventional plate reader technology can be used to measure absorbance of enzyme based chromophores. A method for sorting cells with similar speed to that of conventional FACS may be employed where the electrical charging plates are replaced with high performance electromagnets that allow magnetic based separation. Alternatively, confocal microscopy will allow increased sensitivity but with significant reduction in throughput.

[0168] In a preferred embodiment the assay marker peptide is a naturally fluorescent protein fusion product that includes but is not limited to humanized *Renilla reniformis* green fluorescent protein (hrGFP) with FACS separation. Examples of uncloned GFP molecules useful for practice of the invention have been cited in Cormier, M. J., Hori, K., and Anderson, J. M. (1974) Bioluminescence in Coelenterates. Biochim. Biophys. Acta 346:137-164. In cases where fluorescent signal of the tagged fusion



proteins are of insufficient magnitude to be useful the cells may be probed again with enzyme labeled fluorescence.

[0169] In a further embodiment ELISA and Western blotting may be used to establish correlation curves that increase the accuracy of the protein estimations. Alternatively, RIA and/or immunoprecipitations can be used to establish standard correlation curves of target protein content. Preferably a consistent standard and calibrator set of beads will be developed with a known number of molecules of fluorescent protein bound per bead. These standard beads will allow correlation of fluorescent intensity to molecules equivalent soluble fluorescence.

[0170] In another optional embodiment the expression construct includes a polynucleotide with a negative or positive selection protein for enrichment of the population prior to sorting. Use of the negative or positive selection will remove from the population all cells with no integration of the polynucleotide, for example via antibiotic resistance. This provides for enriched populations of target cells to overcome any relative inefficiency of the gene trapping of genomic control elements. Enrichment of gene trapped cells will include the use of drug selection (e.g., neo<sup>r</sup>, puro<sup>r</sup>, hygro<sup>r</sup>, zeo<sup>r</sup>, HAT<sup>r</sup>), affinity separations to include but not limited to Ab/Ag or Ab/hapten, biotin/streptavidin, glutathione S-transferase (GST) fusion proteins, Polyhistamine fusion proteins (Invitrogen), calmodulin-binding peptide tag (Stratagene), HA epitope tag (YPYDVPDYA), c-myc epitope tag (peptide seq. EQKLISEEDL) (Stratagene), FLAG epitope tag (peptide seq. DYKDDDDK) (Stratagene), V5 epitope (Stratagene), the Linx<sup>TM</sup>. technology {phenyldiboronic acid [PDBA] and salicylhydroxamic acid [SHA] (Invitrogen), adhesion, blocking of adhesion, chemotaxis, block of chemotaxis etc., and/or enrichment by FACS using fluorescent Ab, fluorescent Ag, fluorescent substrates or non-fluorescent substrates that become fluorescent after enzymatic cleavage/activation (A complete listing of common fluorescent probes used for our applications can be found in references: Shapiro, H. M., Practical Flow Cytometry, Third Edition, Wiley-Liss (1994), Robinson, J. P., Handbook of Flow Cytometry Methods, Wiley-Liss (1993); Ormerod, M. G., Flow Cytometry: A Practical Approach, Second Edition, IRL Press (1994); Robinson, J. P., Current Protocols in Cytometry, John Wiley & Sons (2000).

[0171] Alternatively, some applications may use depletion of cells that demonstrate very high levels of protein expression to allow finer fractionation of cells demonstrating lower expression of the marker peptide (e.g., negative selection including but not limited to HSV tk/GCV). This negative selection can be applied before or after a positive selection process.

[0172] According to the invention populations of marker peptide (gene trapping) cells will be sorted by FACS into various levels of expression based on the distribution of number of cells and relative fluorescent intensity. The cells which may be either viable or fixed in preservatives (e.g., Para formaldehyde) will then be sorted into groups based on mean fluorescence intensity. The process is equally efficient with dead fixed and non fixed cell or cells that have been permeabilized and probed with fluorescently labeled Ab or enzyme labeled fluorescent probes to increase sensitivity. Cytometry 23, 46 (1996); J Histochem Cytochem 43, 77 (1995). The process of sorting will yield either mixed populations of cell clones displaying similar mean fluorescence intensity or separated individual cell clones, each of which will display a distribution of fluorescence values which is characteristic of the particular gene trapping event and the particular fusion protein generated between the assay marker gene and the endogenous cellular protein being measured. An example of normal (HMEC) and cancer (MCF7) cell populations were transduced with different vectors (HSG and GTFSO) and sorted in four different fractions displaying different mean fluorescence levels is shown in FIGS. 14A-14D.

[0173] Once the sorting process is completed DNA, RNA, and/or total protein may be extracted and subjected to down stream amplification and/or analysis (although live cells could be returned to culture for further amplification if it is deemed useful to do so).

#### **Sequence Tag Acquisition and Reporting**

[0174] Once cells are separated out according to their fusion protein expression levels, the fusion protein is associated with particular genomic loci by defining the flanking sequences around the integration site of the marker peptide expressing retroviral vectors (e.g., molecular DNA bar code).

[0175] Fractionation of the entire population of cells (with measurable levels of marker protein expression) into subpopulations of cells (each subpopulation comprised of cells with similar levels of marker protein expression) is followed by analysis of integration sites within the cells of a subpopulation. As integration sites are identified (and correlated with a genetic locus) they are assigned the mean subpopulation marker protein level as a measure of relative expression. When complete, all analyzed integration sites/genes will have a relative level of protein expression assigned to them.

[0176] For the purpose of this example, we describe a method for the acquisition of the data needed to assign integration sites to specific genes and to mean marker protein expression levels. It is inclusive of, but not limited to, the examples listed below:

**A. The method of inverse PCR procedure for recovering genomic tags associated to vector or viral integration events**

[0177] As shown in Figure 17, recovery of genetic material from the cells to be analyzed, in this example cellular DNA (inclusive of, but not limited to, cellular DNA since complementary DNA derived from cellular RNA (cDNA) may be used), the composition of which is partially known to the operator by virtue of the inclusion of the sequences encoding the marker peptide. The genetic locus containing the inserted sequence (or producing the RNA containing inserted marker gene sequences) is known as the "tagged gene."

[0178] A method of cleaving said cellular DNA such that inserted DNA (with sequence known to the operator) is cleaved once and flanking cellular DNA of unknown sequence is cleaved again in the regions contiguous to the inserted piece of DNA. Cleavage of the DNA occurs in a fashion generating ends that permit the circularization of DNA fragments producing a molecule with the sequence known to the operator flanking both sides, and continuous with, a variable length of cellular DNA of unknown sequence.

[0179] Primers comprised of sequences drawn from that sequence known to the operator (part of the expression vector) are used in the amplification of unknown

sequences, in this example by polymerase chain reaction (inclusive of, but not limited to, since other means of amplifying sequences as RNA molecules could be used). These primers are selected to bind to the circularized product described previously, in the regions of DNA whose sequence is known to the operator, and prime the synthesis of DNA proceeding in opposing directions causing amplification of the DNA segment of unknown sequence. The product of this reaction will thus contain two terminal segments of DNA sequence known to the operator (as described supra, and an internal DNA segment of unknown sequence. This amplified DNA molecule is known as the captured amplimer.

[0180] The captured amplimer is analyzed for the nucleotide composition of the region whose sequence is unknown to the operator. This may be achieved by any of several methods known to those skilled in the art. Importantly for the invention, the sequence composition is not required to be determined in its entirety, rather a segment adequate to allow identification of its origins by comparison to a sequence database of known composition, e.g. GENBANK.

[0181] The region of the captured amplimer that provides the sequence for comparison is known as the captured sequence. Comparison of the captured sequence to a database can be performed by any of several means known to operators skilled in the art, in this example using BLAST analysis. That portion of the captured sequence that can be matched to the sequence of genetic loci contained in the established database is referred to as the sequence tag.

[0182] The sequence tag, once acquired and annotated with the corresponding genetic locus information and assigned a mean marker protein expression value, then may be used to correlate with a reference cell type. This identifies a potential drug target or a diagnostic indicator of a disease state or other diagnosable difference between the two cell types.

**B. 5' Serial Analysis of Viral Integration (5'SAVI), 3' Serial Analysis of Viral Integration (3'SAVI) and Serial Analysis of Viral Integration (SAVI). Under**

**present invention, three new methods for identifying loci of the marker integration  
may be utilized**

**[0183]** The first method is called 5' Serial Analysis of Viral Integration (5'SAVI). This method is used to identify the sequence of cellular exons fused by the splicing mechanism to the 5' side of the marker gene, which can result in an encoded fusion protein. This method takes advantage of Type IIS restriction enzyme site incorporated at the 5' end of the marker, immediately downstream to the SA signal. Two different versions of this method are outlined in Figures 18A-18D.

**[0184]** FIG. 18A-18B is a schematic depicting one of the possible experimental procedures to carry out the 5'SAVI method. This method starts with purified spliced RNA. Reverse transcription is employed to convert RNA transcripts into a complementary double stranded cDNA (cDNA) following standard methods for full length total cDNA synthesis. This cDNA is then subjected to restriction enzyme digestion with a Type IIS restriction enzyme which will cut the cDNA into the sequences corresponding to the cellular exon ten to twenty bases away from the SD/SA junction depending on which Type IIS restriction enzyme is used. A biotin-labeled primer #1 with a sequence specific for the marker exon is then employed to generate a ssDNA fragment that extends into the cellular exon fused upstream of the marker exon. Collection of this biotin-ssDNA by streptavidin conjugated magnetic beads enriches these specific ssDNA for subsequent DNA terminal transferase reaction. Poly-deoxynucleotide can be added onto these ssDNA as a tail at their 3' end. An oligonucleotide primer complementary to the homopolymeric tail and a second primer #2 nested with respect to primer #1 on the marker gene can therefore be used to amplify by PCR this 3' end of the cellular exon fused to the 5' side of the marker exon. These short tags or amplification fragments from different integrated genes can, by ligation reactions, be made into longer DNA fragments that are subsequently sequenced. Sequencing results of these tags can be used to retrieve the identity from a sequence database.

**[0185]** Figure 18C-18D is a schematic depicting another possible experimental approach to carry out the 5' SAVI method. In this version of the 5'SAVI method, a biotinylated primer #1 specific to the complementary sequence of the marker exon is

used to prime cDNA synthesis by reverse transcriptase. Next, a polynucleotide tail is added to this single stranded cDNA by the enzyme terminal transferase. An oligonucleotide primer complementary to this homopolymeric tail is then used to drive the synthesis of the complementary second DNA strand. Double stranded products are subjected to a type IIS restriction enzyme digestion and the digestion products are purified with magnetic streptavidin beads. An adaptor is ligated to the end generated by the type IIS restriction enzyme and the products are amplified by PCR with a primer corresponding to the adaptor sequence and with a primer #2 specific to the marker gene and nested with respect to primer #1. Amplification products are ligated together into high order polymeric structures, cloned into sequencing vectors and sequenced. Sequencing results of these tags can be used to retrieve the identity from a sequence database.

**[0186]** The methods described above as 5'SAVI can also be applied to retrieve DNA sequence of cellular exons fused by the splicing mechanism to the 3' end of the marker exon sequence. In this case, the marker exon contains a type IIS restriction enzyme at the 3' end of the exon marker immediately followed by a splice donor consensus sequence. Figure 18E-18F depicts the method of 3'SAVI. Upon RNA transcription driven by a cellular promoter or by a heterologous promoter driving the expression of the marker gene, and RNA splicing, the exon marker sequence will generate a fusion to a downstream cellular exon. After total double stranded cDNA synthesis and digestion with a type IIS restriction enzyme, a double stranded DNA adaptor can be ligated to the end generated by the type IIS restriction enzyme. Amplification of fragments containing marker sequences can be accomplished by PCR amplification using a primer #1 specific to the marker exon sequence and a second primer corresponding to the ligated adaptor. After PCR amplification, fragments of equal length can be cloned into high order polymeric structures, cloned into sequencing vectors and sequenced.

**[0187]** Alternatively, the marker gene may contain two recognition sites for a Type IIS restriction endonuclease as shown in FIG. 18G-18H. In this method, the construct that is inserted into the genomic DNA of a cell comprises a marker exon flanked by a splice acceptor and donor consensus sequences, a first Type IIS restriction enzyme recognition (RER) site located upstream of the marker, a second Type IIS RER

site located downstream of the marker, a first non-Type IIS RER site located between the marker and the first Type IIS RER site, a second non-Type IIS RER site located between the marker and the second type IIS RER site. During splicing, assuming that the construct has integrated into an intron in the same orientation as the direction of transcription of the cellular gene, the introns will be removed by the splicing mechanism generating a spliced RNA molecule with an insertion of the marker exon between two cellular exons. Then, mRNA is isolated from the cell, and reverse transcribed into full length double stranded cDNA. The cDNA is subjected to a Type IIS restriction enzyme digestion that recognizes the first and second Type IIS RER sites and thereupon cleaves the cDNA upstream of the first Type IIS RER site and downstream of the second Type IIS RER site such that a cDNA fragment is produced comprising the marker, and portions of the upstream and downstream exon sequences. After digestion with the appropriate Type IIS RE, the fragment is self-ligated generating a circular molecule, where the sequence tags from the upstream and downstream cellular exons are fused in inverse orientation generating a di-tag. This di-tag is then amplified by inverse PCR using marker-specific primers. Following amplification, the fragments are subjected to one or more non-Type IIS REs that recognized the first and second non-Type IIS RER sites and thereupon cleaves the fragments within each of the first and the second non-Type IIS RER sites such that the marker is cleaved away from the fragments. Following non-Type IIS RE digestion, the di-tag fragments are separated and ligated together to form a concatamer, which is then sequenced by appropriate methods. The sequence is then compared to a sequence database such that the protein encoded by the sequence is identified. The structure of vectors described here for use with the SAVI method can also be used to retrieve sequence information from only the upstream or downstream exons by following the above described methods of 5'SAVI or 3'SAVI, respectively. As can be appreciated by one of ordinary skill in the art, this method allows for the quantitative elucidation of protein profile for a given cell coupled with the simultaneous identification of exon boundaries for all gene trapped genes. Since the length of the di-tags corresponding to the upstream and downstream exon boundaries captured by this method is the same for all genes, PCR amplification does not introduce a size bias and the frequency of a di-tag being amplified and sequenced will therefore reflect the relative

abundances of different mRNA transcripts. Therefore, the relative frequencies of di-tags being sequenced can represent the relative levels of transcription and mRNA abundance levels for all identified genes. The combination of different exon boundaries in the ditags from the same gene will provide information about alternative splicing from any given gene.

**[0188]** The SAVI approach, as described above, has several advantages compared to other methods. First, it allows obtaining the sequence of about 8-10 viral integration events per sequencing reaction of contiguous exons, therefore lowering the sequencing costs. Second, it brings the sequence information of contiguous exons, thereby allowing to study alternative splicing and to annotate functional exon boundaries in the genome.

**[0189]** The sequence obtained is analyzed by segmentation into defined lengths (established by the specificities of the enzyme used earlier and is known as a "captured viral integration sequence." Comparison of the captured sequence to the database can be performed by any of several means known to operators skilled in the art, in this example using BLAST analysis. That portion of the captured sequence that can be matched to the sequence of genetic loci contained in the established database is referred to as the "captured SAVI sequence tag."

**[0190]** The "captured SAVI sequence tag" is then annotated with the genetic locus and the mean marker protein expression value and is denoted the "SAVI sequence tag." This information can be used as described earlier.

**[0191]** The application of these technologies yields several important types of information. First, the ability to generate "captured amplimers" (from both genomic DNA and from cDNA) containing sequences of host cell DNA adjacent to the integrated virus provides data that may determine whether the carcinogenic effects of the virus are due to insertional mutagenesis, or more likely, to the expression of viral genes. This information may be particularly relevant in establishing drug regimens to block expression of the viral genes or to block specific changes in cellular gene expression resulting from site-specific HPV integration.

**[0192]** Perhaps more importantly, the ability to quantify the frequencies at which particular sites of viral integration have occurred can provide information on the clonality



of analyzed lesions, potentially even from samples such as Pap smears. This is important because the presence of clonal populations of cells expressing the integrated viral transforming genes should correlate with the development of cancer in humans (as is seen in rodent models).

**[0193]** Similarly, bronchoalveolar carcinoma in humans shows characteristics strikingly similar to tumors induced in sheep by a viral pathogen (Jaagsiekte virus) although no etiologic agent has yet been identified. The application of the methods of the invention in combination with preparation of EST libraries from bronchoalveolar carcinomas and surrounding normal tissues from patients with this disease could provide information on the causative agent in humans providing diagnostic/prognostic markers that can be exploited. Besides these clinical applications, these data acquisition and reporting systems can also be used to study the mechanism of alternative splicing and the gene expressions regulated in alternative splicing manner. The transcriptional levels of genes can also be digitalized and represented by the frequency of genes being captured. The product of these captured gene tags will be used as probes to hybridize a DNA microarray for data validation.

#### **Analysis of Data Obtained From Sequencing of Captured Gene Tags**

**[0194]** In an optimal embodiment the sequence tags and their associated fluorescence/mRNA levels will be used as input. This data will be analyzed concomitantly with publicly and privately available data.

**[0195]** The resultant data can be imported into a proprietary database, or mined directly for quick comparison and pattern matching. This activity will result in a wide variety of information including but not limited to pharmacogenetic targets, pathway and metabolic analysis, comparison of protein expression between and within species, organisms, and cell states.

**[0196]** For the purpose of this application, the term genetic locus is used to specify a particular location within the context of the genome and does not imply a complete transcription or regulatory unit, instead referring to a specific sequence which may comprise all or part of such functional units or sites.

[0197] For the purpose of this application, marker protein concentration refers to the concentration of specific individual protein configurations which result from phosphorylation, acetylation or other structural modifications which affect functional state (*e.g.*, dimerization vs. monomer) in addition to any assumed unmodified peptide arising from the translation of a mRNA.

#### **Data Aggregation Process:**

[0200] The preferred embodiment of the aggregation process consists of four steps. These steps are: 1) matching the tag against its respective protein sequence, 2) associating a concentration or count level with the tag that is derived from data measured in the FACS module, 3) combining all of the available tag protein expression level data obtained from independent experiments performed with a certain cell type or line to obtain a statistical distribution of the protein expression profile for either each particular fusion protein or a composite value for each genetic locus that summarizes the protein expression data of all characterized fusion proteins between the marker gene and the different exons of the protein encoded by said genetic locus, 4) creating tables that represent the information for each tag and the composite information for each genetic locus, 5) statistical evaluations of differences between the distributions of protein expression levels associated to each tag obtained from the reference and test populations of cells. Steps one and two are order independent, meaning that step two can occur before step one without any problems for the process.

[0201] Implementation of step one begins with the receipt of sequence tags (a variable length DNA sequence that will usually be between 16-25 bases) and associated marker protein concentration data (in this example, but not limited to FACS-derived data). Each tag is compared with a database that contains sequence information of the proteins in the organism of interest. Many methods of making this comparison are possible. Potential methods include but are not limited to: hashing algorithms, dynamic programming alignment algorithms (such as Smith-Waterman and Needleman-Wunsch alignment algorithms), suffix trees and arrays, inverted lists, and combined approaches (such as BLAST (combination of hashing and dynamic programming)) as well as any other string alignment algorithm.

**[0202]** The database can consist of annotated or unannotated genomic sequences that find expression in cells as RNA (independent of their translation into protein, e.g., snRNA, scRNAs, RNAs with catalytic activities, etc.), cDNA libraries, EST libraries, protein sequence libraries (including DNA sequences (with or without intronic or exonic sequences) and amino-acid sequences (including primary, secondary and/or tertiary structure information)). Examples of such databases would include the publicly available EST and genomic databases. The end result of the matching step is that every tag becomes associated with a genetic unit (including subdivisions thereof such as specific intron or exon within a transcription unit) or becomes marked as an unknown so that it can be run again as more information about the proteome/transcript becomes known.

**[0203]** In step two, each tag has data associated with it that can be used to derive quantitative values of the corresponding protein expression levels. The derivation of this quantitative value consists of applying a formula (derived early in the process) to the raw fluorescence levels.

**[0204]** Step three consists of taking the individual tag marker protein concentration levels for each individual genetic locus and combining them to create composite value/s for the genetic locus and those closely related loci (e.g., other introns or exons) within a transcription unit. (This value may have various statistical data associated with it including, but not limited to, measures of central tendency, variance.) The derived data represents a statistical profile of the protein concentration dependent upon the properties of the cells being surveyed (e.g., marker protein stability, transport, cellular auto fluorescence, etc.). The derivation process will be optimized for each organism. This optimization may incorporate a wide variety of methods including, but not limited to, comparison of the individual tag's marker protein concentration (in this example FACS-derived but not limited to, e.g. ferrous conjugate and electromagnetically fractionated) with protein levels as measured by other empirical methods (e.g., ELISA, NMR, 2-D gel electrophoresis) and the use of general biological knowledge about protein structure and regulation. These different methods will allow the determination of the accuracy of tags from different regions of proteins on a proteome wide level as well as a more detailed level (for example protein families, super families, proteins with any significant homology, and individual proteins). For those transcription units that produce

no translation products, marker protein concentration values of zero can be assigned. Although these genetic loci will not be useful for the determination of direct protein/genetic locus correlation, integrative studies can determine whether expression of these transcripts correlate with changes in the pattern of expression at other loci and/or participate in more global regulatory phenomena such alterations in the selection of alternative splicing sites, polyadenylation sites, cytoplasmic transport/stability properties, ribosome binding and other translational events, etc.)

**[0205]** Step four consists of placing all of the input and consequently derived information into tables that are suitable for further detailed analysis or loading into a database. The resulting tables will be relational to support the use of analysis tools including, but not limited to, those found in standard OLAP applications, industrial engineering, operations research, artificial intelligence, forecasting techniques, clustering, genetic network inference and pathway analysis. Examples of such data analysis techniques include but are not limited to phylogenetic tree construction, k-means clustering, expectation maximization, self-organizing maps, support vector machines, various public-domain algorithms, as well as mathematical/statistical models like Boolean networks, applications of differential equations, and stochastic and hybrid Petri nets.

**[0206]** Step five consists in the evaluation of statistical differences between reference and test cell lines. For example, to identify candidate genes involved in cancer biology, the method is performed for two tissue types: a test and a reference cell type, such as a normal cell and cancer cells or cell lines from the same tissue. The method of the invention enables quantification of a single protein species per cell in a population of cells from a given cell population line. These cells are then classified into several gates of different protein expression levels. Each experiment is repeated several times, yielding independent protein expression level measurements for each tagged protein. Thus, compilation of all this data will yield a distribution of discrete independent values for each particular fusion protein over several discrete gates for each cell line that was analyzed. Based on the analysis of these distributions as described below, it is possible to rank genes by P-value. One possible way to do this but not limited to, would be to do a randomization test for each gene, where the null hypothesis is that “there is no difference

between the protein level distribution of the gene in the test and reference cell lines". A statistic used to measure these differences is:

$$t = \sum_{i=1}^4 (\alpha_{ij}^c - \alpha_{ij}^n) X_i \text{ where } \alpha_{ij}^x = n_{ij}^x / N_j^x$$

where

$X_i$  = representative fluorescence value for gate  $i$ ,  $i \in \{1, 2, 3, 4 \dots G\}$

$n_{ij}^c$  = number of independent events observed in gate  $i$  for gene  $j$  in the cancer cells or cell line

$n_{ij}^n$  = number of independent events observed in gate  $i$  for gene  $j$  in the normal cells or cell line

$$N_j^c = n_{ij}^c + n_{ij}^n$$

= total independent events observed in gate  $i$  for gene  $j$  over both cells or cell lines

$$N_{cj} = \sum_{i=1}^4 N_j^c$$

= total number of independent events in the cancer cells or cell line over all gates

$$N_j^n = \sum_{i=1}^4 n_{ij}^n$$

= total number of independent events in the normal cell line over all gates

[0207] Under the null hypothesis, it is possible to assign a P-value to the observed value of the statistic using a randomization procedure. We randomly choose  $N_j^c$  events from the pool of  $N_j (= N_j^c + N_j^n)$  total events and consider them to be arising from the test cell line and the rest from the reference cell line. For each such random assignment, the test statistic is calculated and recorded. This procedure is repeated for a large number of iterations (1000-10000) to get a distribution of the test statistic. The P-value of the observed test statistic can be easily calculated as the ratio of the number of points equal to or more extreme than the observed value to the total number of iterations performed.

[0208] In the case where the distribution of protein expression levels is obtained from cloned cells where each clone bears a different exon trapping event, standard statistical methods can be used to evaluate differences between protein expression profiles for a particular fusion protein between the reference and test cell lines, obtained from the fluorescence intensity distribution measured by FACS analysis of the monoclonal population of cells bearing the same protein fusion.

**Screen for Proteins and/or Protein Domains That Interact With a Protein of Interest**  
**By Using Methods of The Instant Invention**

[0209] The original process described herein and in the applications from which the present application claims priority consists in the comparison of protein fusion profiles between two different cell lines or cell lines subjected to different treatments or culture conditions. The protein fusions are obtained by gene trapping of cellular exons by insertion of retroviruses expressing a reporter gene (hrGFP, AcGFP, epitopes, etc) defined as an exon by functional flanking splicing signals, within introns of functional transcriptional units. The expression profile of the different protein fusions is determined by cell sorting of cells into different fractions based on their level of fluorescence and the identity of each gene trapping event is determined by sequencing the exon flanking the reporter exon. Each gene trapping event generates an in-frame fusion with the fluorescent reporter protein, resulting in a measurable signal.

[0210] This process can be modified to include functional restrictions to the generation of the fluorescent signal. This means that the generation of a positive fluorescent signal will require additional mechanisms or events in addition to an in-frame gene trapping with the reporter protein.

[0211] For example, the system is designed so as to require that the fusion protein interacts with other protein or protein domains in order to generate the fluorescent signal. This can be accomplished by having a pair of reporter proteins or protein fragments that reconstitute some type of activity when brought together by protein interacting partners fused to each of these reporter subunits. A condition that must be met is that the interacting subunits of the reporter system do not drive the protein interaction but that the fusion protein domains fused to each subunit do. Examples of generation of fluorescent signal using these two subunit reporter systems are the enzyme complementation assay developed for  $\beta$ -lactamase (PNAS 2002, 99:3469; Nat. Biotech. 2002, 20:619) or fluorescence resonance energy transfer (FRET) between CFP and YFP. Of these two systems  $\beta$ -lactamase is the preferred one because it reconstitutes an enzymatic activity that can be more sensitive than the generation of fluorescence by FRET (See FIG. 25)

**[0212]** This method will provide a profile of fusion protein domains that interact with a target protein of interest (the “bait”). For practical purposes the bait fusion protein should be expressed in excess relative to the expression levels of the fusion protein domains generated by gene trapping. The intensity of the fluorescent signal (or other signal) will result from a combination of the fusion protein levels and the association constant between the interacting protein domains fused to each subunit of the reporter system. This method allows the generation of a library of protein domains able to interact with the target protein of interest as well as protein interaction values (determined by the mean fluorescence intensity) for each of them. In this way, a comparison between two different cell lines or cell lines cultured in different conditions would reflect the differences in protein interactions with a defined protein target.

**[0213]** By way of non-limiting example, a target protein is selected whose function is necessary for tumor growth. The function of this gene may be an important target for development of small molecule drugs to inhibit tumor growth. However, the function of such a protein often involves and depends on interactions with other proteins and therefore an alternative is to target these protein-protein interactions with small molecule drugs. This requires a previous characterization of all the protein domains interacting with the target protein of interest. The characterization of proteins interacting with a particular target has been previously attempted by using yeast two hybrid analysis. However, as this technology reports protein interaction through the transcriptional activation of a reporter gene, the only interactions that can be mapped are those whose fusion products are transported to the nucleus. In contrast, the B-lactamase complementation assay described herein does not require a particular cellular localization of the two fusion products.

**[0214]** In order to perform a characterization of the protein domains that interact with a target protein, the following steps should be performed (FIG. 25).

**[0215]** First, a stable cell line expressing a fusion between the target protein (bait protein) and one of the subunits of the reporter system are constructed (FIG. 25A). Second, gene trapping should be performed on this stable cell line, using retroviral vectors that encode the second subunit of the reporter system in an exon acceptor

configuration as described herein. This will generate a library of gene trapped protein domains fused to the second subunit of the reporter system. Those cells that display a functional protein domain capable of interaction with the bait protein will reconstitute the reporter function and generate a fluorescent signal (FIG. 25B). Each of these cells can be sorted according to their fluorescence levels and analyzed for the identity of the exon fused to the gene trapping subunit (by 5'SAVI, SAVI or 5'RACE) either as a population or after isolation of individual clones.

## **EXAMPLE 1**

### **Results and Descriptions of Vectors**

[0216] A polynucleotide construct (Gene Trap (GT) vector) was constructed with a splicing acceptor (SA) signal of human  $\gamma$ -globin intron #2 in front of humanized renilla green fluorescence protein (hrGFP) to ensure that the hrGFP can be spliced into the exons of trapped genes (FIGS. 4-6). This SA-hrGFP then was inserted into a retroviral vector in an anti-sense orientation to avoid the interference of the transcription function of 5' LTR, furthermore, the 3' LTR of this retroviral vector has been altered with a deletion of U3 region. The duplication of this deletion in 3' LTR into 5' LTR during reverse transcription disables the 5' LTR promoter function. Therefore, this vector becomes a self-inactivation (SIN) vector. For titer analysis and, to ensure the existence of GT vector in retrovirally transduced (infected) cells, a G418 selection marker gene (NeoR) driven by human cytomegalovirus intermediate-early (CMV IE) promoter was inserted in the vector after hrGFP followed by a bovine growth hormone polyadenylation signal (BGH pA). These genes and functional signals were constructed in reverse orientation to LTRs. The gene expression of hrGFP can only occur after this vector is integrated into the downstream of a cellular promoter.

[0217] Similarly, several different exon trapping vectors have been constructed, the structural characteristics of which are outlined in Table 1.



TABLE 1

Table 1: Description of structural features of retroviral vectors for gene trapping

Vector	Viral Backbone	Exon	Selectable Expression Marker Cassette	SA	SD	ATG	Stop Cod	Frame	SIN LTR	pA	Flanking Restriction Site	SAVI Ready	Genomic Ready
pGT-fs0	MoMLV	hrGFP	LTR-Neo	+	-	-	Y	0	N	Y	BsmFI	Y	N
pGT-fs1	MoMLV	hrGFP	LTR-Neo	+	-	-	Y	1	N	Y	BsmFI	Y	N
pGT-fs2	MoMLV	hrGFP	LTR-Neo	+	-	-	Y	2	N	Y	BsmFI	Y	N
pHSG	HIV-1	hrGFP	none	+	-	-	Y	0	Y	Y	PstI	N	N
pHG	HIV-1	hrGFP	none	+	-	-	Y	0	N	Y	PstI	N	N
pHSGEX	HIV-1	hrGFP	none	+	+	-	N	0	Y	Y	Eco57I	Y	N
pHS2GEX2	HIV-1	SG linker-hrGFP	none	+	+	-	N	0	Y	N	Eco57I	Y	N
pHS2GEX3	HIV-1	SG linker-hrGFP- SG linker	none	+	+	-	N	0	Y	N	MmeI	Y	N
pHS2GEX3PA	HIV-1	SG linker-hrGFP- SG linker	PGK- $\alpha$ Gal	+	+	-	N	0	Y	N	MmeI	Y	N
pHS2AcGEX3	HIV-1	SG linker-AcGFP- SG linker	none	+	+	-	N	0	Y	N	MmeI	Y	N
pHS3AcGEX3	HIV-1	SG linker-AcGFP- SG linker	none	+	+	-	N	0	Y	N	MmeI	Y	N
pHS3AcGEX4	HIV-1	SG linker-AcGFP- SG linker	none	+	+	-	N	0	Y	N	MmeI	Y	Y
pHS3AcGEX4PA	HIV-1	SG linker-AcGFP- SG linker	PGK- $\alpha$ Gal	+	+	-	N	0	Y	N	MmeI	Y	Y
pHS3AcGEX4E1bA	HIV-1	SG linker-AcGFP- SG linker	AdE1b- $\alpha$ Gal	+	+	-	N	0	Y	N	MmeI	Y	Y
pHS3AcGEX4E1bNeo	HIV-1	SG linker-AcGFP- SG linker	AdE1b-Neo	+	+	-	N	0	Y	N	MmeI	Y	Y
pGT18	MoMLV	hrGFP	LTR-Neo	+	+	+	N	0	N	N	BsmFI-	N	N
pGT19	MoMLV	hrGFP	LTR-Neo	+	+	+	N	0	N	N	BsmFI-	N	N
pGT22	MoMLV	hrGFP	LTR-Neo	+	+	+	Y	0	N	N	BsmFI	Y	N
pGT-GIG	MoMLV	hrGFP-ires-aGal	LTR-Neo	+	-	-	Y	0	N	Y	BsmFI	Y	N
pGT10	MoMLV	hrGFP	LTR-Neo	+	-	+	Y	0	N	Y	BsmFI	Y	N
pGT11	MoMLV	hrGFP	LTR-Neo	+	-	+	Y	0	N	Y	BsmFI	N	N
pGT12	MoMLV	hrGFP	LTR-Neo	+	+	+	Y	0	N	N	BsmFI	Y	N
pGT13	MoMLV	hrGFP	LTR-Neo	+	+	+	Y	0	N	N	BsmFI	Y	N
pGT14	MoMLV	hrGFP	LTR-Neo	+	+	-	Y	0	N	N	BsmFI	Y	N

Table 1 (Continued)

pGT15	MoMLV	hrGFP	LTR-Neo	+	+	-	Y	0	N	N	BsmFI	Y	N
pGT5	MoMLV	hrGFP	CMV-Neo (AS)	+	-	+	Y	0	Y	Y	FokI	N	N
pGT5AH	MoMLV	hrGFP	CMV-Neo (AS)	+	-	+	Y	0	Y	Y	FokI	N	N
pHS-HA3F	HIV-1	HA epitope (3 copies)	none	+	+	-	N	1,2,0	Y	N	MmeI	Y	N
pHS-HA3XF0	HIV-1	HA epitope (3 copies)	none	+	+	-	N	0	Y	N	MmeI	Y	N
pHS-HA3XF1	HIV-1	HA epitope (3 copies)	none	+	+	-	N	1	Y	N	MmeI	Y	N
pHS-HA3XF2	HIV-1	HA epitope (3 copies)	none	+	+	-	N	2	Y	N	MmeI	Y	N
pHS-V5His	HIV-1	V5, His epitopes	none	+	+	-	Y	0	Y	N	MmeI	Y	N
pFS0-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	0	N	Y	MmeI	Y	N
pFS1-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	1	N	Y	MmeI	Y	N
pFS2-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	2	N	Y	MmeI	Y	N
pYS0-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	0	N	Y	MmeI	Y	N
pYS1-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	1	N	Y	MmeI	Y	N
pYS2-Neo	MoMLV	Neo	LTR-Hyg	+	-	-	Y	2	N	Y	MmeI	Y	N
pGT-8	MoMLV	hrGFP	CMV-Neo (AS)	+	-	+	Y	1	Y	Y	FokI	Y	N
pGT-9	MoMLV	hrGFP	CMV-Neo (AS)	+	-	+	Y	2	Y	Y	FokI	Y	N

**[0218]** These vectors combine several structural features such as being based on either MoMLV or HIV-1 backbones, the use of different reporter marker genes (hrGFP, AcGFP, Neo, HA and V5 epitopes), the presence of either one or both splice acceptor and splice donor consensus sequences, the presence or absence of selectable markers, exon trapping markers in different translational reading frames and different restriction sites flanking the marker in order to allow for identification of the flanking sequence tags by using different methodologies described before, such as 5'RACE, 3'RACE, 5' SAVI, 3' SAVI, SAVI or inverse PCR. More specifically, as summarized in Table 1, these vectors are either based on the MoMLV or HIV-1 viral backbones; they contain different markers for exon trapping such as hrGFP, AcGFP, hrGFP-ires galactosyltransferase, Neo, or the epitopes HA or V5. Some of these vectors contain a selectable marker to select for transduced cells such as  $\alpha$ -galactosyltransferase, Neomycin or Hygromycin resistance genes, and the expression of these markers is driven by either the viral LTR promoter or an internal promoter such as Adenovirus Elb, CMV or PGK. All vectors contain the reporter exon preceded by a splice acceptor consensus sequence at the 5' end of the reporter sequence, and some of them are also flanked by a splice donor consensus sequence at the 3' end of the reporter exon. In some vectors the ATG translation initiation codon has been mutated to avoid reporting integrations that occur in the 5' UTR of cellular genes. The marker gene is translated in either frames 0, 1 or 2 as indicated.  $\alpha$ SIN LTR makes reference to a deletion of critical enhancer/promoter sequences in the 3' U3 region of the 3' LTR. pA indicates when a polyadenylation sequence is present in the vector downstream of the reporter gene. The restriction sites for type IIS restriction enzymes that flank either one end or both ends of the insertion exon is also indicated in Table I. Also it is indicated whether these vectors are amenable to analysis of flanking sequence tags by the SAVI procedure or by rescue of genomic insertion points by restriction digestion of genomic DNA, re-ligation and bacterial transformation.

**[0219]** We have been able to show successful gene trapping with our GT vectors in murine fibroblasts, NIH3T3 (FIGS. 11 and 12) and PA317 cells (FIG. 8A-B), human lung cancer cells (FIG. 10A-B). Fluorescence-activated cell sorting (FACS) has been employed to separate the gene-trapped cell population which shows green fluorescence after 488 nm UV light excitation. The enrichment of these gene trapping events were

performed by cell sorting machine (Altra Cell Sorter, Beckman Coulter Co, Miami Fla., USA) showed that 95% of cell population (FIG. 8A-B) were fluorescence positive and gene trapped since the hrGFP expression can only occur after this hrGFP gene was integrated downstream of a cellular promoter. FIG. 14A-D shows examples of normal (HMEC) and cancer (MCF7) breast cell lines transduced with vectors pGTFS0 or pHSG which were sorted by FACS into different gates according to their levels of expression of the fluorescent protein marker fused to different cellular proteins by means of exon trapping events. Furthermore, in theory, these hrGFP molecules should be a fusion protein with a cellular protein in frame after splicing occurred to join cellular exons and hrGFP together. This hypothesis of splicing and fusion protein has been demonstrated by a construct pGT5Z (FIG. 6) using a Zeocin-resistance protein to hrGFP after splicing and translation (FIG. 7). RNA transcripts of hrGFP in gene-trapped population were also detected by RT-PCR method (FIG. 9). These results demonstrate that gene trapping events can be monitored on translational level by FACS and transcription level by RT-PCR analysis in this experiment.

**[0220]** One important aspect of the invention is the high throughput platform to sort gene-trapped cells by using FACS which can sort 15,000 cells/second. The quantity and stability of trapped gene product, which is fused to hrGFP and becomes a hrGFP fusion protein, can be determined by the intensities of hrGFP in the cells in FACS analysis (FIGS. 11 and 12). The invention can therefore be applied to determine the cellular protein levels at high throughput manner and monitor most of the pathways of gene expression altered by the causes of diseases, such as cancer, viral infection, drug treatment and gene transfer in gene therapy or gene transfer research. Other reporter genes can be used to replace the hrGFP gene, in other experiment, rodent  $\alpha$ -1,3-galactosyltransferase gene, which is not expressed in a human cell, was used to demonstrate that gene trapping can be achieved by simple plasmid transfection in up to 1% of the population (FIG. 13C) as well as by retroviral vector infection (FIG. 13B). The results of this experiment are shown in FIGS. 21 and 22.

**[0221]** FIG. 21 is a depiction of a successful gene trapping in pGT5A-transfected PA317 cells. NcoI restriction site located at the 5' end of hrGFP marker gene and an

EcoRI at the Oligo-dA primer were used as cloning sites for gene trapped sequence into a sequencing vector which was digested with NcoI and EcoRI. After BLAST searching against mouse EST database in GenBank, the sequence trapped by pGT5A is a 99% match to a high mobility group protein, HMGI-C, a nuclear phosphoprotein that contains three short DNA-binding domains (AT-hooks) and a highly acidic C-terminus.

[0222] Interest in this protein has recently been stimulated by three observations: the expression of the gene is cell-cycle regulated, the gene is rearranged in a number of tumors of mesenchymal origin and mice that have both HMGI-C alleles disrupted exhibit the pygmy phenotype. These observations suggest a role for HMGI-C in cell growth, more specifically, during fetal growth since the protein is normally only expressed in embryonic tissues. It is likely that the HMGI-C protein acts as an architectural transcription factor, regulating the expression of one or more genes that control embryonic cell growth. Since HMGI-C binds to the minor groove at AT-rich DNA this interaction could be a target for minor groove chemotherapeutic agents in the treatment of sarcomas expressing the rearranged gene. As can be seen, the invention successfully identified a potential oncogene with a demonstration of high translation level of this gene product indicated by high intensity of hrGFP fusion protein in FACS analysis (FIG. 8).

[0223] FIG. 22 is a depiction of gene trapping of an exon with unknown biological function in pGT5A-transfected PA317 cells. NcoI restriction site located at the 5' end of hrGFP marker gene and an EcoRI at the oligo-dA primer were used as cloning sites for gene trapped sequence into a sequencing vector which was digested with NcoI and EcoRI. After BLAST searching against the EST database in GenBank, the sequence trapped by pGT5A is 95% match to a NCI\_CGAP\_Li9 Mus musculus cDNA clones, BF539247.1/BF533319.1, which have been found in the cDNA libraries from salivary gland and liver. As can be seen the invention successfully identified a gene without known biological function, but with a known high-level of protein production indicated by the fusion protein of this gene product and hrGFP in FACS analysis in FIG. 8. These results indicate that this invention can correlate the translation level of genes to some other unknown or undefined DNA sequences for potential new discoveries of genes or targets responsible for diseases or cancers.

**[0224]** Additional examples of sequence tags obtained by gene trapping with the vectors pHSG and pGT10 from cells sorted into subgroups C, D, E and F according to their fluorescence values (see FIG. 14A-D) are presented in Table 2.

TABLE 2

Table 2: Examples of genes trapped by the method of the invention along with mean fluorescence intensity

Vector	Gate	Accession	CDS - start	CDS - end	Protein Length	Insertion Point	aa Fused to N ter	Fraction of Protein Captured	Definition
HSG	C	NM_052987.1	91	1035	315	90	0	0	cyclin-dependent kinase (CDC2-like) 10 (CDK10), transcript variant 2, mRNA
HSG	C	NM_032733.1	116	235	40	133	6	15	hypothetical protein MGC12679 (MGC12679), mRNA
HSG	C	NM_014892.1	186	4001	1272	215	10	1	KIAA1116 protein (KIAA1116), mRNA
HSG	C	NM_012075.1	286	1995	570	321	12	2	Conserved gene telomeric to alpha globin cluster (CGTHBA), mRNA
HSG	C	XM_167532.1	194	562	123	238	15	12	similar to agrin (LOC245749), mRNA
HSG	C	XM_088476.4	209	1429	407	265	19	5	endoplasmic reticulum resident protein 44 kDa (ERp44), mRNA
HSG	C	XM_041484.3	72	461	130	128	19	15	similar to tektin 1 (LOC91917), mRNA
HSG	C	NM_002827.2	175	1482	436	237	21	5	protein tyrosine phosphatase, non-receptor type 1 (PTPN1), mRNA
HSG	C	NM_005339.2	78	680	201	140	21	10	huntingtin interacting protein 2 (HIP2), mRNA
HSG	C	NM_002898.1	17	1240	408	82	22	5	RNA binding motif, single stranded interacting protein 2 (RBMS2), mRNA
HSG	C	NM_017876.1	64	999	312	138	25	8	hypothetical protein FLJ20552 (FLJ20552), mRNA
HSG	C	NM_000937.2	387	6299	1971	479	31	2	polymerase (RNA) II (DNA directed) polypeptide A (220kD) (POLR2A), mRNA
HSG	C	NM_005526.1	161	1750	530	277	39	7	heat shock transcription factor 1 (HSF1), mRNA
HSG	C	NM_003883.2	56	1342	429	193	46	11	histone deacetylase 3 (HDAC3), mRNA
HSG	C	XM_051343.3	33	2882	950	191	53	6	BDG-29 protein (BDG-29), mRNA
HSG	C	NM_000135.1	32	4399	1456	220	63	4	Fanconi anemia, complementation group A (FANCA), mRNA
HSG	C	NM_014403.1	50	958	303	247	66	22	sialyltransferase 7D (alpha-N-acetylneuraminy-2,3-beta-galactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase (SIAT7D), mRNA

Table 2 (Continued)

HSG	C	NM 014280.1	8	802	265	220	71	27	DnaJ (Hsp40) homolog, subfamily C, member 8 (DNAJC8), mRNA
HSG	C	NM 015332.1	483	1145	221	701	73	33	KIAA1068 protein (KIAA1068), mRNA
HSG	C	NM 006908.2	1	579	193	225	75	39	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1), transcript variant Rac1, mRNA
HSG	C	NM 017730.1	25	1275	417	282	86	21	hypothetical protein FLJ20259 (FLJ20259), mRNA
HSG	C	NM 002719.1	89	1633	515	352	88	17	protein phosphatase 2, regulatory subunit B (B56), gamma isoform (PPP2R5C), mRNA
HSG	C	NM 014963.1	216	4316	1367	494	93	7	KIAA0963 protein (KIAA0963), mRNA
HSG	C	Hs#S4284146	233	2563	777	541	103	13	cDNA FLJ23733 fis, clone HEP14786
HSG	C	NM 014757.2	264	3314	1017	578	105	10	mastermind-like 1 (Drosophila) (MAML1), mRNA
HSG	C	NM 006816.1	1	1071	357	315	105	29	chromosome 5 open reading frame 8 (C5orf8), mRNA
HSG	C	NM 001499.1	88	2067	660	408	107	16	GLE1 RNA export mediator-like (yeast) (GLE1L), mRNA
HSG	C	XM 050236.6	482	1900	473	814	111	23	leukocyte receptor cluster (LRC) member 4 (LENG4), mRNA
HSG	C	XM 087781.2	23	850	276	370	116	42	cofactor required for Sp1 transcriptional activation, subunit 8 (34kD) (CRSP8), mRNA
HSG	C	XM 087782.1	9	401	131	356	116	89	similar to p37 TRAP/SMCC/PC2 subunit (LOC220792), mRNA
HSG	C	NM 001755.1	12	560	183	410	133	73	core-binding factor, beta subunit (CBFB), transcript variant 2, mRNA
HSG	C	NM 014287.2	1	3669	1223	402	134	11	pM5 protein (PM5), mRNA
HSG	C	NM 001664.1	152	733	194	559	136	70	ras homolog gene family, member A (ARHA), mRNA
HSG	C	NM 007274.1	150	1268	373	563	138	37	cytosolic acyl coenzyme A thioester hydrolase (HBACH), mRNA
HSG	C	NM 012127.1	152	2692	847	583	144	17	Cip1-interacting zinc finger protein (CIZ1), mRNA
HSG	C	NM 005968.2	231	2423	731	668	146	20	heterogeneous nuclear ribonucleoprotein M (HNRPM), transcript variant 1, mRNA
HSG	C	NM 016329.1	326	2926	867	778	151	17	RU1 (RU1), mRNA
HSG	C	NM 001417.1	1	1836	612	477	159	26	eukaryotic translation initiation factor 4B (EIF4B), mRNA
HSG	C	NM 003563.1	158	1282	375	637	160	43	speckle-type POZ protein (SPOP), mRNA
HSG	C	NM 005016.2	89	1189	367	592	168	46	poly(rC) binding protein 2 (PCBP2), transcript variant 1, mRNA
HSG	C	NM 000135.1	32	4399	1456	553	174	12	Fanconi anemia, complementation group A (FANCA), mRNA
HSG	C	NM 007353.1	1	1146	382	525	175	46	guanine nucleotide binding protein (G protein) alpha 12 (GNA12), mRNA



Table 2 (Continued)

HSG	C	NM_001876.1	77	2398	774	631	185	24	carnitine palmitoyltransferase 1, liver (CPT1A), nuclear gene encoding mitochondrial protein, mRNA
HSG	C	NM_017921.1	133	1959	609	786	218	36	hypothetical protein FLJ20657 (NPL4), mRNA
HSG	C	NM_006600.1	91	1086	332	831	247	74	nuclear distribution gene C homolog (A. nidulans) (NUDC), mRNA
HSG	C	NM_030791.1	33	1358	442	806	258	58	sphingosine-1-phosphatase (LOC81537), mRNA
HSG	C	NM_005032.2	88	1980	631	1074	329	52	plastin 3 (T isoform) (PLS3), mRNA
HSG	C	NM_012469.1	100	2925	942	1122	341	36	chromosome 20 open reading frame 14 (C20orf14), mRNA
HSG	C	NM_138570.1	346	2688	781	1476	377	48	hypothetical protein MGC15523 (MGC15523), mRNA
HSG	C	NM_006575.2	321	2861	847	1505	395	47	mitogen-activated protein kinase kinase kinase 5 (MAP4K5), mRNA
HSG	C	NM_016355.2	27	1394	456	1262	412	90	hqp0256 protein (LOC51202), mRNA
HSG	C	NM_006282.1	43	1506	488	1347	435	89	serine/threonine kinase 4 (STK4), mRNA
HSG	C	NM_000918.2	45	1571	509	1490	482	95	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55) (P4HB), mRNA
HSG	C	NM_000135.1	32	4399	1456	1657	542	37	Fanconi anemia, complementation group A (FANCA), mRNA
HSG	C	NM_012308.1	107	3595	1163	1939	611	53	F-box and leucine-rich repeat protein 11 (FBXL11), mRNA
HSG	C	NM_003074.1	55	3369	1105	1890	612	55	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 (SMARCC1), mRNA
HSG	C	XM_114045.1	28	3009	994	2091	688	69	KIAA0090 protein (KIAA0090), mRNA
HSG	C	NM_003816.1	79	2538	820	2376	766	93	a disintegrin and metalloproteinase domain 9 (meltrin gamma) (ADAM9), mRNA
HSG	C	XM_046305.2	7	3654	1216	2316	770	63	KIAA1205 protein (KIAA1205), mRNA
HSG	C	XM_029470.2	56	4315	1420	2392	779	55	chromosome 17 open reading frame 31 (C17orf31), mRNA
HSG	C	NM_000208.1	107	4255	1383	2788	894	65	insulin receptor (INSR), mRNA
HSG	C	NM_004830.1	120	4226	1369	2897	926	68	cofactor required for Sp1 transcriptional activation, subunit 3 (130kD) (CRSP3), mRNA
HSG	C	NM_003127.1	103	7521	2473	5820	1906	77	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1), mRNA
HSG	D	XM_046390.3	238	2853	872	246	3	0	zinc finger protein ZFP100 (ZFP100), mRNA
HSG	D	XM_113649.1	146	1900	585	175	10	2	similar to KIAA1730 protein (LOC196102), mRNA
HSG	D	NM_016076.1	59	640	194	100	14	7	CGL-146 protein (LOC51029), mRNA
HSG	D	NM_005805.1	200	1132	311	247	16	5	26S proteasome-associated pad1 homolog (POH1), mRNA

Table 2 (Continued)

HSG	D	NM_002074.2	333	1355	341	389	19	6	guanine nucleotide binding protein (G protein), beta polypeptide 1 (GNB1), mRNA
HSG	D	NM_002898.1	17	1240	408	82	22	5	RNA binding motif, single stranded interacting protein 2 (RBMS2), mRNA
HSG	D	NM_001769.1	52	738	229	117	22	10	CD9 antigen (p24) (CD9), mRNA
HSG	D	XM_165637.1	68	1633	522	136	23	4	karyopherin alpha 3 (importin alpha 4) (KPNA3), mRNA
HSG	D	NM_024835.1	48	2141	698	140	31	4	C3HC4-type zinc finger protein (LZK1), mRNA
HSG	D	NM_001102.2	184	2862	893	288	35	4	actinin, alpha 1 (ACTN1), mRNA
HSG	D	NM_003100.1	30	1589	520	137	36	7	sorting nexin 2 (SNX2), mRNA
HSG	D	NM_014294.2	92	1216	375	214	41	11	translocating chain-associating membrane protein (TRAM), mRNA
HSG	D	NM_032350.1	80	664	195	208	43	22	hypothetical protein MGC11257 (MGC11257), mRNA
HSG	D	NM_006937.1	91	378	96	243	51	53	SMT3 suppressor of mif two 3 homolog 2 (yeast) (SMT3H2), mRNA
HSG	D	NM_012297.2	121	1569	483	297	59	12	Ras-GTPase activating protein SH3 domain-binding protein 2 (KIAA0660), mRNA
HSG	D	NM_005389.1	74	757	228	265	64	28	protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1), mRNA
HSG	D	NM_001326.1	132	2285	718	356	75	10	cleavage stimulation factor, 3&apos; pre-RNA, subunit 3, 77kD (CSTF3), mRNA
HSG	D	XM_047011.2	50	1216	389	295	82	21	protein O-fucosyltransferase 1 (POFUT1), mRNA
HSG	D	NM_022780.2	99	1274	392	383	95	24	hypothetical protein FLJ13910 (FLJ13910), mRNA
HSG	D	XM_032350.6	328	1428	367	612	95	26	similar to dJ462O23.1 (novel protein) (LOC90529), mRNA
HSG	D	NM_003406.1	85	822	246	378	98	40	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), mRNA
HSG	D	NM_002817.1	70	1200	377	378	103	27	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13 (PSMD13), mRNA
HSG	D	NM_030579.1	37	477	147	357	107	73	cytochrome b5 outer mitochondrial membrane precursor (CYB5-M), mRNA
HSG	D	NM_007372.1	18	2147	710	386	123	17	RNA helicase-related protein (RNAHP), mRNA
HSG	D	NM_017812.1	138	821	228	506	123	54	hypothetical protein FLJ20420 (FLJ20420), mRNA
HSG	D	NM_004368.1	28	957	310	417	130	42	calponin 2 (CNN2), mRNA
HSG	D	NM_005877.2	132	2513	794	524	131	16	splicing factor 3a, subunit 1, 120kD (SF3A1), mRNA
HSG	D	NM_005147.1	32	1474	481	460	143	30	tumorous imaginal discs (Drosophila) homolog (TID1), mRNA

Table 2 (Continued)

HSG	D	NM 014676.1	96	3656	1187	527	144	12	pumilio homolog 1 ( <i>Drosophila</i> ) (PUM1), mRNA
HSG	D	NM 005973.3	219	1694	492	686	156	32	papillary renal cell carcinoma (translocation-associated) (PRCC), mRNA
HSG	D	NM 003491.1	98	805	236	568	157	67	N-acetyltransferase, homolog of <i>S. cerevisiae</i> ARD1 (ARD1), mRNA
HSG	D	NM 006386.2	309	2261	651	309	167	26	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 17 (72kD) (DDX17), transcript variant 1, mRNA
HSG	D	NM 138639.1	683	1687	335	1363	227	68	BCL2-like 12 (proline rich) (BCL2L12), transcript variant 1, mRNA
HSG	D	NM 006709.2	11	3643	1211	874	288	24	HLA-B associated transcript 8 (BAT8), transcript variant
HSG	D	NM 014230.1	1	1860	620	954	318	51	NG36/G9a, mRNA
HSG	D	NM 004544.1	22	1089	356	1020	333	94	signal recognition particle 68kD (SRP68), mRNA
HSG	D	NM 002973.1	163	4101	1313	1335	391	30	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10 (42kD) (NDUFA10), mRNA
HSG	D	NM 002973.1	163	4101	1313	1335	391	30	spinocerebellar ataxia 2 (olivopontocerebellar ataxia 2, autosomal dominant, ataxin 2) (SCA2), mRNA
HSG	D	NM 004196.4	113	3991	1293	1291	393	30	DKFZP434C212 protein (DKFZP434C212), mRNA
HSG	D	NM 000245.1	189	4415	1409	1388	400	28	met proto-oncogene (hepatocyte growth factor receptor) (MET), mRNA
HSG	D	NM 014672.1	360	2063	568	1586	409	72	KIAA0391 gene product (KIAA0391), mRNA
HSG	D	NM 006311.1	241	7563	2441	1647	469	19	nuclear receptor co-repressor 1 (NCOR1), mRNA
HSG	D	NM 133335.1	139	4236	1366	1812	558	41	Wolf-Hirschhorn syndrome candidate 1 (WHSC1), transcript variant 3, mRNA
HSG	D	NM 007331.1	495	2384	630	2168	558	89	Wolf-Hirschhorn syndrome candidate 1 (WHSC1), transcript variant 8, mRNA
HSG	D	NM 024536.2	19	1587	523	2016	666	127	hypothetical protein FLJ22678 (FLJ22678), mRNA
HSG	D	NM 005085.1	95	6367	2091	2134	680	33	nucleoporin 214kD (CAIN) (NUP214), mRNA
HSG	D	NM 004958.2	80	7729	2550	3109	1010	40	FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), mRNA
HSG	D	NM 000937.2	387	6299	1971	3626	1080	55	polymerase (RNA) II (DNA directed) polypeptide A (220kD) (POLR2A), mRNA
HSG	D	NM 004953.1	369	4559	1397	4034	1222	87	eukaryotic translation initiation factor 4 gamma, 1 (EIF4G1), mRNA
HSG	E	NM 021090.1	248	3844	1199	250	1	0	myotubularin related protein 3 (MTMR3), mRNA
HSG	E	NM 000899.1	184	1005	274	198	5	2	KIT ligand (KITLG), mRNA

Table 2 (Continued)

HSG	E	XM 085473.2	99	1046	316	119	7	2	similar to KIAA0251 hypothetical protein (LOC146452), mRNA
HSG	E	NM 003901.1	178	1884	569	204	9	2	sphingosine-1-phosphate lyase 1 (SGPL1), mRNA
HSG	E	NM 004906.1	125	580	152	154	10	7	Wilms&apos; tumour 1-associating protein (KIAA0105), mRNA
HSG	E	NM 012308.1	107	3595	1163	148	14	1	F-box and leucine-rich repeat protein 11 (FBXL11), mRNA
HSG	E	XM 113712.1	48	539	164	89	14	9	similar to citrate synthase precursor (LOC196407), mRNA
HSG	E	NM 007242.2	105	1544	480	161	19	4	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 19 (DBP5 homolog, yeast) (DDX19), mRNA
HSG	E	NM 032117.1	29	646	206	97	23	11	GAJ protein (GAJ), mRNA
HSG	E	NM 004860.2	228	2249	674	308	27	4	fragile X mental retardation, autosomal homolog 2 (FXR2), mRNA
HSG	E	NM 012412.2	172	558	129	252	27	21	histone H2A.F/Z variant (H2AV), transcript variant 1, mRNA
HSG	E	XM 059929.1	6	320	105	89	28	27	similar to RIKEN cDNA 311003A22 (LOC137886), mRNA
HSG	E	NM 001696.2	135	815	227	233	33	15	ATPase, H+ transporting, lysosomal 31kD, V1 subunit E (ATP6V1E), mRNA
HSG	E	NM 001102.2	184	2862	893	288	35	4	actinin, alpha 1 (ACTN1), mRNA
HSG	E	NM 032141.1	29	1705	559	142	38	7	hypothetical protein DKFZp434K1421 (DKFZP434K1421), mRNA
HSG	E	NM 000402.1	471	2018	516	590	40	8	glucose-6-phosphate dehydrogenase (G6PD), nuclear gene encoding mitochondrial protein, mRNA
HSG	E	NM 003095.1	116	376	87	244	43	49	small nuclear ribonucleoprotein polypeptide F (SNRPF), mRNA
HSG	E	NM 024071.2	35	739	235	172	46	20	hypothetical protein MGC2550 (MGC2550), mRNA
HSG	E	NM 005868.2	120	476	119	263	48	40	BET1 homolog (S. cerevisiae) (BET1), mRNA
HSG	E	NM 004128.1	120	869	250	278	53	21	general transcription factor IIF, polypeptide 2 (30kD subunit) (GTF2F2), mRNA
HSG	E	NM 006628.2	125	463	113	292	56	50	cyclic AMP phosphoprotein, 19 kD (ARPP-19), mRNA
HSG	E	NM 001761.1	44	2404	787	214	57	7	cyclin F (CCNF), mRNA
HSG	E	NM 003086.1	376	4785	1470	552	59	4	small nuclear RNA activating complex, polypeptide 4, 190kD (SNAPC4), mRNA
HSG	E	XM 042708.4	18	2531	838	197	60	7	hypothetical protein FLJ14743 (FLJ14743), mRNA
HSG	E	NM 005778.1	149	2596	816	331	61	7	RNA binding motif protein 5 (RBM5), mRNA
HSG	E	NM 002337.1	14	1087	358	217	68	19	low density lipoprotein-related protein-associated protein 1 (alpha-2-macroglobulin receptor-associated protein 1) (LRPAP1), mRNA

Table 2 (Continued)

HSG	E	NM 014366.1	171	1853	561	380	70	12	putative nucleotide binding protein, estradiol-induced (E2IG3), mRNA
HSG	E	XM 058647.2	12	383	124	233	74	60	similar to CG1577 gene product (LOC122704), mRNA
HSG	E	XM 027330.6	1636	2703	356	1863	76	21	S164 protein (S164), mRNA
HSG	E	NM 014463.1	30	338	103	257	76	74	Lsm3 protein (LSM3), mRNA
HSG	E	NM 006321.1	145	1626	494	399	85	17	ariadne homolog 2 (Drosophila) (ARIH2), mRNA
HSG	E	NM 002969.2	310	1413	368	564	85	23	mitogen-activated protein kinase 12 (MAPK12), mRNA
HSG	E	XM 058850.3	77	664	196	334	86	44	LOC124801 (LOC124801), mRNA
HSG	E	NM 032631.1	55	2067	671	342	96	14	hypothetical protein MGC2641 (MGC2641), mRNA
HSG	E	NM 000786.2	332	1861	510	622	97	19	cytochrome P450, 51 (lanosterol 14-alpha-demethylase) (CYP51), mRNA
HSG	E	NM 003406.1	85	822	246	378	98	40	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), mRNA
HSG	E	NM 015133.1	1	4029	1343	318	106	8	mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3), mRNA
HSG	E	NM 030579.1	37	477	147	357	107	73	cytochrome b5 outer mitochondrial membrane precursor (CYB5-M), mRNA
HSG	E	NM 020151.1	554	1441	296	877	108	36	START domain containing 7 (STARD7), mRNA
HSG	E	NM 004397.2	367	1785	473	702	112	24	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 6 (RNA helicase, 54kD) (DDX6), mRNA
HSG	E	NM 005721.2	216	1472	419	551	112	27	ARP3 actin-related protein 3 homolog (yeast) (ACTR3), mRNA
HSG	E	XM 034571.2	35	607	191	370	112	59	similar to HN1 like (LOC90861), mRNA
HSG	E	NM 003127.1	103	7521	2473	465	121	5	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1), mRNA
HSG	E	NM 021138.1	55	1560	502	420	122	24	TNF receptor-associated factor 2 (TRAF2), mRNA
HSG	E	XM 119331.1	1	717	239	384	128	54	LOC204827 (LOC204827), mRNA
HSG	E	NM 000194.1	86	742	219	469	128	58	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) (HPRT1), mRNA
HSG	E	NM 012218.1	1	2685	895	411	137	15	interleukin enhancer binding factor 3, 90kD (ILF3), mRNA
HSG	E	XM 053717.5	9	2447	813	431	141	17	proline and glutamic acid rich nuclear protein (PELP1), mRNA
HSG	E	NM 014947.1	313	2181	623	756	148	24	KIAA1041 protein (KIAA1041), mRNA
HSG	E	NM 030662.1	264	1466	401	713	150	37	mitogen-activated protein kinase 2 (MAP2K2), mRNA
HSG	E	NM 021734.2	137	1099	321	595	153	48	solute carrier family 25 (mitochondrial deoxynucleotide carrier), member 19 (SLC25A19), mRNA

Table 2 (Continued)

HSG	E	NM_012308.1	107	3595	1163	592	162	14	F-box and leucine-rich repeat protein 11 (FBXL11), mRNA
HSG	E	NM_080423.1	195	1256	354	689	165	47	protein tyrosine phosphatase, non-receptor type 2 (PTPN2), transcript variant 3, mRNA
HSG	E	NM_015497.1	43	1710	556	546	168	30	DKFZP564G2022 protein (DKFZP564G2022), mRNA
HSG	E	NM_000135.1	32	4399	1456	553	174	12	Fanconi anemia, complementation group A (FANCA), mRNA
HSG	E	XM_046408.5	203	2071	623	730	176	28	KIAA1041 protein (KIAA1041), mRNA
HSG	E	NM_032410.1	164	2320	719	694	177	25	hook3 protein (HOOK3), mRNA
HSG	E	NM_002695.2	23	655	211	589	189	90	polymerase (RNA) II (DNA directed) polypeptide E (25kD) (POLR2E), mRNA
HSG	E	NM_015456.2	16	1758	581	612	199	34	cofactor of BRCA1 (COBRA1), mRNA
HSG	E	NM_014892.1	186	4001	1272	791	202	16	KIAA1116 protein (KIAA1116), mRNA
HSG	E	NM_006024.3	101	2344	748	712	204	27	Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1), mRNA
HSG	E	NM_000918.2	45	1571	509	668	208	41	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55) (P4HB), mRNA
HSG	E	NM_007322.1	11	1714	568	703	231	41	RAN binding protein 3 (RANBP3), transcript variant RANBP3-d, mRNA
HSG	E	NM_006311.1	241	7563	2441	972	244	10	nuclear receptor co-repressor 1 (NCOR1), mRNA
HSG	E	NM_080598.2	202	1488	429	936	245	57	HLA-B associated transcript 1 (BAT1), transcript variant 2, mRNA
HSG	E	NM_005207.1	510	1421	304	1286	259	85	v-crk sarcoma virus CT10 oncogene homolog (avian)-like (CRKL), mRNA
HSG	E	NM_032408.1	285	4736	1484	1175	297	20	bromodomain adjacent to zinc finger domain, 1B (BAZ1B), transcript variant 2, mRNA
HSG	E	NM_006599.1	319	4914	1532	1269	317	21	nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5), mRNA
HSG	E	NM_014287.2	1	3669	1223	963	321	26	pM5 protein (PM5), mRNA
HSG	E	NM_001034.1	195	1364	390	1211	339	87	ribonucleotide reductase M2 polypeptide (RRM2), mRNA
HSG	E	NM_001338.2	102	1199	366	1118	339	93	coxsackie virus and adenovirus receptor (CXADR), mRNA
HSG	E	NM_006819.1	63	1694	544	1085	341	63	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein) (STIP1), mRNA
HSG	E	NM_032364.2	401	1570	390	1438	346	89	cytokine induced protein 29 kDa (CIP29), mRNA
HSG	E	NM_018282.1	294	1367	358	1343	350	98	paraspeckle protein 1 (PSP1), mRNA

Table 2 (Continued)

HSG	E	NM 138570.1	346	2688	781	1476	377	48	hypothetical protein MGC15523 (MGC15523), mRNA
HSG	E	NM 080797.1	201	1835	545	1361	387	71	death associated transcription factor 1 (DATF1), transcript variant 3, mRNA
HSG	E	NM 018979.1	1	7149	2383	1311	437	18	protein kinase, lysine deficient 1 (PRKWINK1), mRNA
HSG	E	NM 018128.1	44	2014	657	1369	442	67	hypothetical protein FLJ10534 (FLJ10534), mRNA
HSG	E	XM 026977.4	70	7503	2478	1875	602	24	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1), mRNA
HSG	E	XM 165591.1	108	2225	706	2186	693	98	OLIGOSACCHARYL TRANSFERASE STT3 SUBUNIT HOMOLOG (B5) (INTEGRAL MEMBRANE PROTEIN 1) (LOC219846), mRNA
HSG	E	NM 015356.1	10	4902	1631	2124	705	43	scribble (SCRIB), mRNA
HSG	E	NM 006662.1	211	9126	2972	2436	742	25	Snf2-related CBP activator protein (SRCAP), mRNA
HSG	E	NM 004859.1	173	5200	1676	2464	764	46	clathrin, heavy polypeptide (Hc) (CLTC), mRNA
HSG	E	NM 003127.1	103	7521	2473	3108	1002	41	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1), mRNA
HSG	E	XM 088459.4	109	4494	1462	3522	1138	78	KIAA0310 gene product (KIAA0310), mRNA
HSG	E	NM 130474.1	192	4631	1480	3707	1172	79	MAP-kinase activating death domain (MADD), transcript variant 6, mRNA
HSG	E	NM 002956.1	133	4416	1428	3693	1187	83	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein) (RSN), mRNA
HSG	E	NM 015356.1	10	4902	1631	3612	1201	74	scribble (SCRIB), mRNA
HSG	E	NM 001270.1	164	5293	1710	4024	1287	75	chromodomain helicase DNA binding protein 1 (CHD1), mRNA
HSG	E	NM 001273.1	90	5828	1913	3968	1293	68	chromodomain helicase DNA binding protein 4 (CHD4), mRNA
HSG	F	NM 021177.1	219	506	96	221	1	1	chromosome 6 open reading frame 28 (C6orf28), mRNA
HSG	F	Hs#S3323401	168	2189	674	179	4	1	Similar to kinesin family member C1, clone MGC:1202 IMAGE:3506669, mRNA, complete cds
HSG	F	NM 003379.2	118	1878	587	129	4	1	villin 2 (ezrin) (VIL2), mRNA
HSG	F	XM 031251.4	104	1030	309	118	5	2	glycoprotein, synaptic 2 (GPSN2), mRNA
HSG	F	NM 004859.1	173	5200	1676	214	14	1	clathrin, heavy polypeptide (Hc) (CLTC), mRNA
HSG	F	NM 003079.2	126	1361	412	176	17	4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 (SMARCE1), mRNA
HSG	F	NM 033534.1	183	686	168	236	18	11	cell division cycle 2-like 2 (CDC2L2), transcript variant 7, mRNA

Table 2 (Continued)

HSG	F	NM_002074.2	333	1355	341	389	19	6	guanine nucleotide binding protein (G protein), beta polypeptide 1 (GNB1), mRNA
HSG	F	NM_001839.1	84	1073	330	140	19	6	calponin 3, acidic (CNN3), mRNA
HSG	F	NM_003142.1	73	1299	409	138	22	5	Strogren syndrome antigen B (autoantigen La) (SSB), mRNA
HSG	F	NM_003169.2	208	3471	1088	282	25	2	suppressor of Ty 5 homolog (S. cerevisiae) (SUT5H), mRNA
HSG	F	XM_119330.1	1	384	128	81	27	21	LOC204826 (LOC204826), mRNA
HSG	F	NM_006391.1	108	3224	1039	191	28	3	RAN binding protein 7 (RANBP7), mRNA
HSG	F	NM_003324.1	46	1374	443	138	31	7	tubby like protein 3 (TULP3), mRNA
HSG	F	XM_052065.3	193	2112	640	291	33	5	hypothetical protein DKFZp547A023 (DKFZp547A023), mRNA
HSG	F	NM_032921.1	652	1179	176	753	34	19	hypothetical protein MGC15875 (MGC15875), mRNA
HSG	F	XM_072156.3	394	2346	651	501	36	6	solute carrier family 23 (nucleobase transporters), member 1 (SLC23A1), mRNA
HSG	F	NM_000927.2	425	4264	1280	541	39	3	ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), mRNA
HSG	F	XM_005588.5	63	971	303	185	41	14	hypothetical protein FLJ20651 (FLJ20651), mRNA
HSG	F	NM_014783.1	722	3793	1024	850	43	4	KIAA0013 gene product (ARHGAP11A), mRNA
HSG	F	NM_002096.1	179	1732	518	310	44	8	general transcription factor IIF, polypeptide 1 (74kD subunit) (GTF2F1), mRNA
HSG	F	NM_002213.2	307	2706	800	462	52	7	integrin, beta 5 (ITGB5), mRNA
HSG	F	NM_014808.1	118	3282	1055	300	61	6	KIAA0793 gene product (KIAA0793), mRNA
HSG	F	NM_005898.2	349	2319	657	555	69	11	membrane component, chromosome 11, surface marker 1 (M11S1), mRNA
HSG	F	NM_006869.1	181	1305	375	393	71	19	centaurin, alpha 1 (CENTA1), mRNA
HSG	F	XM_047600.2	31	1995	655	249	73	11	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47) (TCF3), mRNA
HSG	F	NM_004965.1	151	453	101	405	85	84	high-mobility group (nonhistone chromosomal) protein 14 (HMG14), mRNA
HSG	F	NM_007372.1	18	2147	710	281	88	12	RNA helicase-related protein (RNAHP), mRNA
HSG	F	NM_013242.1	257	838	194	532	92	47	similar to mouse Gif3 or D. melanogaster transcription factor IIB (AF093680), mRNA
HSG	F	NM_001755.1	12	560	183	293	94	51	core-binding factor, beta subunit (CBFB), transcript variant 2, mRNA
HSG	F	NM_003908.1	117	1118	334	413	99	30	eukaryotic translation initiation factor 2, subunit 2 (beta, 38kD) (EIF2S2), mRNA



Table 2 (Continued)

HSG	F	NM_006410.2	122	850	243	424	101	42	HIV-1 Tat interactive protein 2, 30 kD (HTATIP2), mRNA
HSG	F	NM_003932.2	75	1184	370	389	105	28	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein) (ST13), mRNA
HSG	F	NM_007276.3	112	663	184	441	110	60	chromobox homolog 3 (HP1 gamma homolog, Drosophila) (CBX3) transcript variant 1, mRNA
HSG	F	XM_034571.2	35	607	191	370	112	59	similar to HN1 like (LOC90861), mRNA
HSG	F	NM_006148.1	76	861	262	432	119	45	LIM and SH3 protein 1 (LASP1), mRNA
HSG	F	NM_138765.1	53	592	180	421	123	68	BCL2-associated X protein (BAX), transcript variant sigma, mRNA
HSG	F	NM_014977.1	328	4353	1342	705	126	9	likely ortholog of mouse acinus (ACN), mRNA
HSG	F	NM_005500.1	46	1086	347	429	128	37	SUMO-1 activating enzyme subunit 1 (SAE1), mRNA
HSG	F	XM_056082.3	1	1953	651	390	130	20	KIAA1936 protein (KIAA1936), mRNA
HSG	F	XM_031324.5	182	3214	1011	574	131	13	hypothetical protein FLJ22206 (FLJ22206), mRNA
HSG	F	NM_007375.2	135	1379	415	536	134	32	TAR DNA binding protein (TARDBP), mRNA
HSG	F	NM_002276.1	33	1235	401	452	140	35	keratin 19 (KRT19), mRNA
HSG	F	NM_006170.1	1	2568	856	474	158	18	nucleolar protein 1 (NOL1), mRNA
HSG	F	NM_014570.2	39	1589	517	515	159	31	ADP-ribosylation factor GTPase activating protein 1 (ARFGAP1), mRNA
HSG	F	XM_033161.4	288	917	210	776	163	78	HSPC133 protein (HSPC133), mRNA
HSG	F	NM_003286.2	247	2544	766	753	169	22	topoisomerase (DNA) I (TOP1), mRNA
HSG	F	NM_002583.1	239	1267	343	760	174	51	PRKC, apoptosis, WT1, regulator (PAWR), mRNA
HSG	F	XM_046822.2	140	4315	1392	664	175	13	KIAA1814 protein (KIAA1814), mRNA
HSG	F	NM_133439.1	373	1290	306	903	177	58	transcriptional adaptor 2 (ADA2 homolog, yeast)-like (TADA2L), transcript variant 2, mRNA
HSG	F	NM_080702.1	261	3641	1127	812	184	16	HLA-B associated transcript 3 (BAT3), transcript variant 2, mRNA
HSG	F	XM_012616.6	300	917	206	890	197	96	CGI-148 protein (LOC51030), mRNA
HSG	F	NM_003816.1	79	2538	820	684	202	25	a disintegrin and metalloproteinase domain 9 (meltrin gamma) (ADAM9), mRNA
HSG	F	NM_012423.2	23	634	204	628	202	99	ribosomal protein L13a (RPL13A), mRNA
HSG	F	NM_015560.1	56	2938	961	679	208	22	optic atrophy 1 (autosomal dominant) (OPA1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA
HSG	F	NM_005968.2	231	2423	731	860	210	29	heterogeneous nuclear ribonucleoprotein M (HNRPM), transcript variant 1, mRNA

Table 2 (Continued)

HSG	F	NM 006845.2	55	2232	726	717	221	30	kinesin-like 6 (mitotic centromere-associated kinesin) (KNSL6), mRNA
HSG	F	NM 006819.1	63	1694	544	734	224	41	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein) (STIP1), mRNA
HSG	F	NM 003089.1	681	2525	615	1358	226	37	small nuclear ribonucleoprotein 70kD polypeptide (RNP antigen) (SNRP70), mRNA
HSG	F	NM 012399.2	40	855	272	807	256	94	phosphatidylinositol transfer protein, beta (PITPNB), mRNA
HSG	F	NM 004395.2	144	2093	650	914	257	40	drebrin 1 (DBN1), transcript variant 1, mRNA
HSG	F	NM 053275.1	111	1064	318	902	264	83	ribosomal protein, large, P0 (RPLP0), transcript variant 2, mRNA
HSG	F	NM 001090.1	95	2518	808	895	267	33	ATP-binding cassette, sub-family F (GCN20), member 1 (ABCF1), mRNA
HSG	F	XM 042287.3	35	2950	972	937	301	31	HSPC055 protein (HSPC055), mRNA
HSG	F	NM 003942.1	66	2384	773	971	302	39	ribosomal protein S6 kinase, 90kD, polypeptide 4 (RPS6KA4), mRNA
HSG	F	NM 022455.2	19	8109	2697	945	309	11	nuclear receptor binding SET domain protein 1 (NSD1), mRNA
HSG	F	NM 016451.1	179	3040	954	1135	319	33	coatamer protein complex, subunit beta (COPB), mRNA
HSG	F	NM 005826.2	91	1992	634	1107	339	53	heterogeneous nuclear ribonucleoprotein R (HNRPR), mRNA
HSG	F	NM 004537.2	125	1300	392	1183	353	90	nucleosome assembly protein 1-like 1 (NAP1L1), mRNA
HSG	F	NM 016448.1	103	2295	731	1227	375	51	RA-regulated nuclear matrix-associated protein (RAMP), mRNA
HSG	F	NM 002613.1	81	1751	557	1205	375	67	3-phosphoinositide dependent protein kinase-1 (PDPK1), mRNA
HSG	F	XM 084653.4	18	1769	584	1361	448	77	radixin (RDX), mRNA
HSG	F	NM 014452.2	24	1991	656	1532	503	77	tumor necrosis factor receptor superfamily, member 21 (TNFRSF21), mRNA
HSG	F	NM 001746.1	90	1868	593	1607	506	85	calnexin (CANX), mRNA
HSG	F	NM 021930.2	24	2489	822	1781	586	71	Rad50-interacting protein 1 (FLJ11785), mRNA
HSG	F	NM 014278.1	55	2574	840	1866	604	72	heat shock protein (hsp110 family) (APG-1), mRNA
HSG	F	NM 005066.1	86	2209	708	1900	605	85	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) (SFPQ), mRNA
HSG	F	NM 015904.2	142	3804	1221	1983	614	50	translation initiation factor IF2 (IF2), mRNA
HSG	F	NM 003760.2	257	5014	1586	2221	655	41	eukaryotic translation initiation factor 4 gamma, 3 (EIF4G3), mRNA

Table 2 (Continued)

HSG	F	XM_046305.2	7	3654	1216	2316	770	63	KIAA1205 protein (KIAA1205), mRNA
HSG	F	NM_005054.1	78	5375	1766	2774	899	51	RAN binding protein 2-like 1 (RANBP2L1), transcript variant 1, mRNA
HSG	F	NM_003127.1	103	7521	2473	2880	926	37	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1), mRNA
HSG	F	NM_002375.2	158	3616	1153	3223	1022	89	microtubule-associated protein 4 (MAP4), transcript variant 1, mRNA
HSG	F	NM_000937.2	387	6299	1971	3626	1080	55	polymerase (RNA) II (DNA directed) polypeptide A (220kD) (POLR2A), mRNA
HSG	F	XM_040067.2	158	5119	1654	4924	1589	96	transcription factor 20 (AR1) (TCF20), mRNA
GT10	C	NM_001743.3	69	518	150	71	1	1	calmodulin 2 (phosphorylase kinase, delta)
GT10	C	NM_000968.2	57	1340	428	59	1	0	ribosomal protein L4
GT10	C	NM_000969.2	63	956	298	65	1	0	ribosomal protein L5
GT10	C	NM_005620.1	121	438	106	123	1	1	S100 calcium binding protein A11 (calgizzarin)
GT10	C	NM_005659.1	79	1110	344	81	1	0	ubiquitin fusion degradation 1-like
GT10	C	NM_003761.2	54	356	101	56	1	1	vesicle-associated membrane protein 8 (endobrevin)
GT10	C	NM_005139.1	47	1018	324	61	5	2	annexin A3
GT10	C	NM_000980.2	36	566	177	53	6	3	ribosomal protein L18a
GT10	C	NM_003340.2	166	609	148	189	8	5	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
GT10	C	NM_004671.1	1	1866	622	24	8	1	Protein inhibitor of activated STAT X
GT10	C	NM_015859.1	405	1535	377	434	10	3	general transcription factor IIA, 1 (37kD and 19kD subunits)
GT10	C	NM_005882.2	23	1210	396	58	12	3	macrophage erythroblast attacher
GT10	C	NM_014617.2	109	633	175	147	13	7	crystallin, gamma A
GT10	C	XM_040280.2	110	1270	387	166	19	5	KIAA1724 protein
GT10	C	NM_003680.1	1	1587	529	57	19	4	tyrosyl-tRNA synthetase
GT10	C	NM_002047.1	519	2576	686	578	20	3	glycyl-tRNA synthetase
GT10	C	NM_033103.1	28	2085	686	96	23	3	rhophilin-like protein
GT10	C	NM_004496.1	88	1509	474	159	24	5	hepatocyte nuclear factor 3, alpha
GT10	C	NM_013943.1	198	959	254	269	24	9	chloride intracellular channel 4
GT10	C	NM_018163.1	12	926	305	89	26	9	hypothetical protein FLJ10634
GT10	C	NM_024082.1	34	729	232	120	29	13	transmembrane gamma-carboxyglutamic acid protein 3
GT10	C	NM_006791.1	132	1103	324	218	29	9	MORF-related gene 15
GT10	C	NM_031459.1	177	1619	481	266	30	6	sestrin 2

Table 2 (Continued)

GT10	C	NM_016424.2	154	1452	433	252	33	8	cisplatin resistance-associated overexpressed protein
GT10	C	XM_039714.2	106	1020	305	207	34	11	zinc finger protein 364
GT10	C	NM_023938.1	165	1970	602	269	35	6	hypothetical protein MGC2742
GT10	C	NM_000690.1	37	1587	517	147	37	7	aldehyde dehydrogenase 2 family (mitochondrial)
GT10	C	NM_004844.1	64	1341	426	174	37	9	SH3-domain binding protein 5 (BTK-associated)
GT10	C	NM_001263.1	437	1771	445	553	39	9	CDP-diacylglycerol synthase (phosphatidate cytidyltransferase) 1
GT10	C	NM_022143.2	141	2102	654	266	42	6	NAG14 protein
GT10	C	NM_015959.1	50	940	297	238	63	21	CGI-31 protein
GT10	C	NM_001730.2	312	1685	458	572	87	19	Kruppel-like factor 5 (intestinal)
GT10	C	NM_003276.1	205	2289	695	483	93	13	thymopoietin
GT10	C	NM_138962.1	183	1169	329	494	104	32	musashi homolog 2 (Drosophila)
GT10	C	NM_006106.2	250	1614	455	570	107	24	Yes-associated protein 1, 65 kD
GT10	C	NM_002473.1	1	5883	1961	333	111	6	myosin, heavy polypeptide 9, non-muscle
GT10	C	NM_030756.1	308	2098	597	688	127	21	transcription factor 7-like 2 (T-cell specific, HMG-box)
GT10	C	NM_032872.1	168	1820	551	626	153	28	NADPH oxidase-related, C2 domain-containing protein
GT10	C	NM_001379.1	238	5088	1617	837	200	12	DNA (cytosine-5-)-methyltransferase 1
GT10	C	NM_003645.1	223	2085	621	927	235	38	fatty-acid-Coenzyme A ligase, very long-chain 1
GT10	C	NM_005412.2	22	1536	505	1404	461	91	serine hydroxymethyltransferase 2 (mitochondrial)
GT10	C	NM_001270.1	164	5293	1710	3988	1275	75	chromodomain helicase DNA binding protein 1

Mean fluorescence intensity for each protein is in arbitrary units and is indicated in the Gate column as follows:

Gate C = 75000

Gate D = 125000

Gate E = 175000

Gate F = 230000

**[0225]** This table shows the subgroup of cells from where each particular tag was obtained (Gate), their GenBank accession number (Accession), the position of the coding sequence start (CDS Start) and stop (CDS Stop) nucleotides within the mRNA, the number of aminoacids of the protein encoded by that particular mRNA (Protein Length), the position within that mRNA where the 5' end of the GFP marker exon was inserted (Insertion Point), the number of amino acids of the tagged cellular protein fused to the 5' end of the GFP marker protein (aa Fused to N-ter), the fraction of the protein fused to GFP (Fraction of Protein Captured) and the identity name of the tagged cellular protein (Definition). Other types of information associated to each data record not displayed in the abridged version of this table are: information on reported gene annotation, function, and chromosomal location, with the additional ability to link directly to NCBI web pages regarding such information. Also, information about measures of quality control for each collected tag are obtained in the process. Measures of internal quality control reported for the viewer include: quality of the sequencing run, base by base; and the sequence quality of the validation sequence which must precede all captured data tags. External quality control measures available to the viewer include a determination of the degree of local identity between data tag and the matched NCBI RefSeq cDNA, as well as the calculation of the length of the match.

**[0226]** FIGS. 22 and 23 represent compilations of genes captured, sequenced and annotated using the described method with several gene-trapping vectors. FIG. 22 displays the allocation of all captured proteins among different subcellular localizations as a percentage of the total number of captured proteins. The graph in FIG. 22 represents a total of 1271 data records and at least 109 subcellular localization descriptions which have been classified into one of 8 subheadings: unknown, cytoplasm, nucleus, membrane, cytoskeletal, organelle, extracellular and peripheral membrane. The vectors displayed include, HSG, pGT10, pGTFS0, -1, and -2 (combined and denoted as FS012). The general trend of the subcellular distribution of the endogenous proteins identified by the chimeric fusion products appears consistent among different gene-trapping vectors in two different target cell lines, MCF-7 and HCT-15. FIG. 23 displays the putative functions of all collected gene-trapped products among the listed vectors and cell lines. The graph in FIG. 23 represents 1271 data records and at least 233 categories placed in one of 16

major subheadings for protein function. The data is displayed as the percentage of captured proteins contained in each subheading for protein function. A consistent trend in the distribution of captured proteins over various functional subheadings can be observed among the different gene-trapping vectors and target cell lines. The compilation of data records obtained by the described method can therefore potentially describe the global picture of the active proteome. Expansion of this dataset to include all detectable chimeric fusion products can be used to make comparisons regarding function and cell localization among individual proteins of the proteome within different tissues, cell types and phenotypes. The method of the present invention can also be used to compare the translational regulation of gene products among cancer and normal cells by comparing the dataset on protein versus conventional RNA microarrays. Additionally, the method may be used to characterize global changes to cell/tissue phenotypes by the analysis of shifts in the distribution pattern of the proteome among different subcellular localizations or protein functions.

**[0227]**           The foregoing description is presented by way of illustration and is not intended to limit the scope of the present invention as set out in the appended claims.

**[0228]**           All of the references cited herein are incorporated by reference in their entirety.